Chromatin Changes at Sites of DNA Double-Strand Breaks

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Summary

Eukaryotic cells are constantly exposed to a multitude of genotoxic agents of either exogenous sources such as exposure to ultraviolet components of the sunlight, or of endogenous sources such as metabolically produced reactive oxygen species. The resulting lesions are a constant threat to the stability of the genome. DNA double-strand breaks (DSBs) are among the most cytotoxic types of DNA damage. They are induced by ionizing radiation or clastogens and can be generated by replication over a single-strand break. If left unrepaired they can lead to cell death or gross chromosomal aberrations, and promote the onset of diseases associated with genomic instability such as cancer.

In mammalian cells, a global signalling network – called the DNA damage response (DDR) – has evolved that senses the DNA lesions and regulates appropriate responses such as DNA repair, transient or permanent cell cycle arrest, apoptosis or senescence as well as induction of certain transcriptional programs. In response to DSBs, the MRE11/RAD50/NBS1 (MRN) complex is involved in the recruitment and activation of the kinases ATM and DNA-PKcs that phosphorylate various target proteins. Among those is the histone variant H2AX, a natural component of chromatin that occurs in about 7-15% of all nucleosomes. The adaptor protein MDC1 directly binds to phosphorylated H2AX (γH2AX) and mediates recruitment and retention of other DDR factors in chromatin domains flanking the lesion. MDC1 is constitutively phosphorylated by casein kinase 2, which was shown to be required for the recruitment of the MRN complex to damaged chromatin via its subunit NBS1. NBS1 contains two phospho-specific interaction modules at its N-terminus, a FHA domain and a BRCT tandem domain. In this study, we show that both of these domains participate in the interaction with MDC1. Phospho-binding activities of both domains are essential for accumulation of the MRN complex in chromatin adjacent to DSBs *in vivo*. Surprisingly though, only mutation in the FHA domain, but not mutation in the BRCT domains, yields a G2/M checkpoint defect. Moreover, we did not detect increased radiosensitivity upon mutation of either the FHA or the BRCT tandem domain. Therefore, we concluded that MDC1-dependent retention of the MRN complex in the γH2AX-containing chromatin is not essential for a proper G2/M checkpoint activation, nor is it required for normal survival after ionizing radiation.

Furthermore, we suggest that the FHA domain of NBS1 may have additional as yet unidentified binding partners that mediate G2/M checkpoint activation in response to DSBs. Therefore, we set out to search for such additional interaction partners. In fission yeast, NBS1 interacts with Ctp1 via the FHA domain, and this interaction is required for survival upon various DNA damaging agents. However, our preliminary data reveal that CtIP, the human orthologue of Ctp1, is rather not a direct binding partner of the FHA domain of human NBS1.

Zusammenfassung

Zellen sind ständig Substanzen ausgesetzt, die die DNA beschädigen. Diese können von der Umwelt (z.B. Sonnenstrahlung) oder durch den Organismus (z.B. freie Sauerstoffradikale) produziert werden. Die Gefahr besteht, dass die so entstanden DNA Schäden das Genom des Organismus verändern. Besonders Doppelstrangbrüche der DNA sind eine der grössten Bedrohungen. Sie entstehen durch die Einwirkung von ionisierender Strahlung oder auch durch die Replikation eines DNA Fragmentes mit einem Einzelstrangbruch. Falls Doppelstrangbrüche nicht repariert werden, können sie zum Zelltod oder zu Translokationen führen. Sie können auch zu Krankheiten wie Krebs führen.

Menschliche Zellen besitzen spezialisierte Mechanismen zur Erkennung und Reparatur von DNA Schäden, sowie zur Aktivierung von Signalkaskaden, die das Fortschreiten des Zellzyklus hemmen, bestimmte Transkriptionsprogramme einleiten oder den programmierten Zelltod auslösen. Entstehen solche Doppelstrangbrüche, dann hilft der MRE11/RAD50/NBS1 (MRN) Komplex die beiden Kinasen ATM und DNA-PKcs zu aktivieren. Diese phosphorylieren dann unzählige Proteine, eines davon ist die Histone-Variante H2AX, die in 7-15% der Nukleosomen vorkommt. Das Adaptorprotein MDC1 bindet phosphoryliertes H2AX und führt zu weiterer Akkumulierung verschiedener Reparaturfaktoren im Chromatin in der Nähe des DNA Bruches. MDC1 wird von der Casein Kinase 2 phosphoryliert, was zur Rekrutierung vom MRN Komplex führt via seine Untereinheit NBS1. Am N-terminalen Ende von NBS1 sind zwei phosphospezifische Interaktionsdomänen lokalisiert, die FHA Domäne und die BRCT tandem Domäne. In dieser Studie zeigen wir, dass beide Domänen für die Interaktion zwischen MDC1 und dem MRN Komplex verantwortlich sind und somit auch beide für die Akkumulierung des MRN Komplexes im Chromatin gebraucht werden. Interessanterweise führt aber nur die Mutation einer konservierten Aminosäure in der FHA Domäne zu einem Defekt der Aktivierung des G2/M Zellzykluskontrollpunktes, nicht aber die Mutation in der BRCT Domäne von NBS1. Desweiteren konnten wir zeigen, dass Zellen mit den mutierten Domänen nicht radiosensitiver sind als Wildtypzellen. Daraus folgt, dass die Rekrutierung des MRN Komplexes nicht essenziell ist für die Aktivierung des G2/M Kontrollpunktes.

Zusätzlich schlagen wir vor, dass NBS1 mit der FHA Domäne noch mit anderen Proteinen interagiert und dadurch den G2/M Kontrollpunkt aktiviert. Wir haben auch schon einige Kandidaten getestet. Es ist zum Beispiel bekannt, dass in Hefe die Interaktion zwischen Ctp1 und der FHA Domäne von NBS1 für die DNA Reparatur benötigt wird. Unsere ersten Ergebnisse zeigen jedoch, dass CtIP, das orthologe Protein in Menschenzellen, wahrscheinlich nicht mit der FHA Domäne von NBS1 interagiert.

Table of contents

1 Chromatin changes at sites of DSBs

1.1 DNA damage, genomic stability and cancer

DNA damage arises constantly through exposure to a multitude of genotoxic agents and as a result of intracellular metabolism. The sources of DNA damage can be exogenous such as exposure to ultraviolet (UV) components of sunlight, ionizing radiation (IR) or genotoxic chemicals taken up by our body. Furthermore, they can also be endogenous since by-products of the cellular metabolism such as reactive oxygen species (ROS) of oxidative respiration may harm the DNA. In addition, DNA damage may also arise by inaccurate replication. The DNA lesions resulting from these sources are a constant threat to the stability of the genome.

The preservation of the genetic information is essential for all living organisms. During cell division the whole genome has to be faithfully replicated to give rise to two identical daughter cells. If left unrepaired or improperly repaired, DNA damage may lead to mutations, smaller deletions or even gross chromosomal rearrangements such as translocations as well as large interstitial or terminal deletions with de novo telomere addition. All of these events may ultimately alter the genetic information. Cells that acquire chromosomal aberration have to be taken out of the proliferative pool by either induction of senescence or programmed cell death (apoptosis). If a cell can escape these surveillance mechanisms, it can acquire more abnormal behaviour and eventually transform into a cancer cell. Thus, DNA damage – if not repaired properly – may be life threatening. Genomic instability is not only involved in the development of cancer but it is a hallmark of cancer cells (Figure 1.1; Negrini et al., 2010).

Cells have evolved surveillance mechanisms to protect the genome and maintain its integrity. The damages are detected and repaired, cells with DNA lesions arrest their cell cycles, and if the load of damages is too big to be repaired cells induce apoptosis. All these processes belong to the DNA damage response (DDR). Genes that give rise to proteins that are involved in genomic surveillance, such as DNA repair genes, are sometimes referred to as 'caretakers'. Defects, deletions or mutations of such genes may lead to predisposition to cancer and may enhance tumorigenesis, which is often the case in hereditary cancer syndromes. Affected individuals inherit a defective allele of a caretaker gene. The incidental mutation of the second allele in a somatic cell promotes tumour survival and disease progression. Examples for such inherited mutations in caretaker genes are the mutations in the BRCA1 or BRCA2 genes which results in increased incidences of breast and ovarian cancers or the mutations in the subunit NBS1 of the MRN complex that display high incidence of developing lymphomas, and mutations in the two helicases WRN and BLM that are known to be responsible for two syndromes connected to lymphomas and leukaemia.

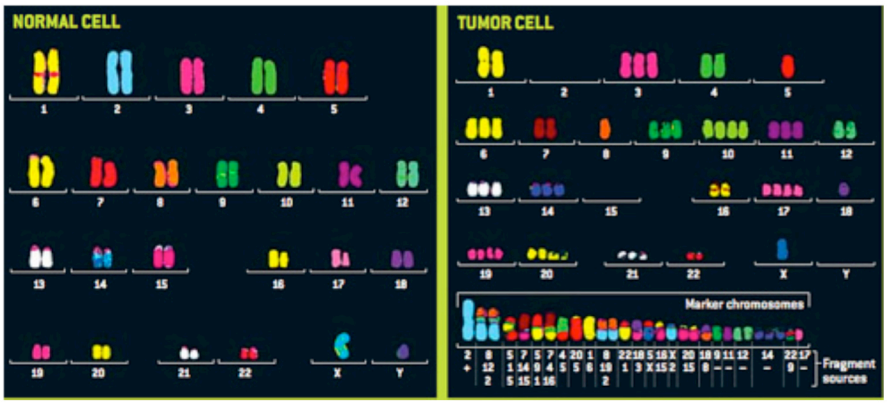

Figure 1.1 Karyotypes of a tumour cell compared to a normal cell. A normal human cells contains a set of 23 pairs of chromosomes whereas tumour cells often acquire extra chromosomes or loose chromosomes (so-called aneuploidy). Besides that, many cancer cells feature “mosaic chromosomes” that are composed of parts of different chromosomes. These are usually generated by translocation events (www.physorg.com/news102179512.html; P. Duesberg, UC Berkeley).

The DDR was observed to be constitutively activated in precancerous lesions and it was proposed that this may constitute a natural barrier for tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). This constitutively activated DDR is thought to be caused by ’replication stress’ that results from oncogene activation (Halazonetis et al., 2008). According to this hypothesis, the replication stress is most pronounced at loci known as common fragile sites that are difficult to replicate and thus are prone to break formation. In addition, shortened telomeres also activate a DDR similar to the one activated by DSBs (d’Adda di Fagagna et al., 2003; Takai et al., 2003). As long as the DDR machinery works properly in these cells, cell cycle arrest and induction of senescence and/or apoptosis prevents tumour formation. However, the constitutively activated DDR leads to selective pressure to disable the genes that regulate these processes. Once these are lost, the doors are open for genome instability to accumulate. However, whether genome instability per se is driving malignant transformation is not yet clear. It could just simply be that the ‘right’ genes are lost. This may explain why so many cancers have lost p53, one of the key regulators of cell cycle progression and apoptosis (Bartek et al., 2007).

Understanding the processes of the DDR is important for cancer therapy. DNA damaging agents are commonly used in cancer treatments. Cancer cells are usually more sensitive to these agents than normal cells, because they actively divide and secondly because they have disabled one or several genome surveillance pathways. In addition, cancer cells suffer from several other types of stress including hypoxia, metabolic stress, mitotic stress and proteotoxic stress. Cancer cells must constantly deal with this stress, which makes them more dependent on certain stress-coping mechanisms such as DDR pathways. Thus, cancer

cells may be more sensitive to inhibition of one or the other DDR pathway than normal cells. Consequently, DDR pathways represent new cancer therapy targets.
Investigating the mechanisms of DNA damage response and repair pathways is important to improve the understanding of cancer development as well as to increase the number of possible therapeutic targets.

1.2 The DNA damage response

In mammalian cells, a global signalling network called the DNA damage response (DDR) has evolved that senses the DNA lesions and initiates appropriate cellular responses. The spectrum of DNA lesions is quite variable and ranges from relatively harmless single base or nucleotide modifications to highly toxic lesions such as DNA double strand breaks (DSBs). Most lesions of the former type are quickly repaired and do not induce a global DDR (reviewed in Hoeijmakers 2001). Only when the repair of such smaller lesions is impaired, and single-strand breaks (SSBs) that are induced during the repair mechanism are transformed by replication into DSBs, these lesions might trigger a global DDR. In contrast, DSBs and long stretches of single stranded DNA are two pathogenic DNA structures that have the potential to trigger a global DDR (Wyman and Kanaar, 2006).
In the last decade a vast amount of factors involved in the DDR were identified. They can be classified into four different subtypes (reviewed in Zhou and Elledge, 2000). Enzymes or protein complexes that recognize the DNA damage itself are called sensor proteins. They bind to the aberrant DNA structures and are responsible for activating the signalling cascade. The sensors typically bind and activate protein kinases that are recruited by them to the damaged sites. These protein kinases belong to the family of phosphatidylinositole(3)-like kinase (PIKK) that includes ATM, ATR and DNA-PKcs. These protein kinases phosphorylate and activate the transducer kinases CHK1 and CHK2 that amplify the signal and activate the effector proteins by phosphorylation (Figure 1.2). Alternatively, PIKKs can directly phosphorylate and activate effector proteins. Effector proteins translate the signal into the various cellular responses to the DNA damage. These include regulation of repair pathways, activation of cell cycle checkpoints, induction of apoptosis or senescence and activation of certain transcriptional programs. Recently, the growing importance of mediator or adaptor proteins in this processes has emerged. This family of proteins act as molecular ‘matchmakers’ in that they associate with several other DDR proteins such as PIKKs, transducer kinases or effector proteins, often in a manner that is dependent on post-translational modification, and thus help to organize protein complexes. This is a simplified model and recent findings suggest that rather than being a simple linear signalling pathway, the DDR is a complex network of several interacting pathways.

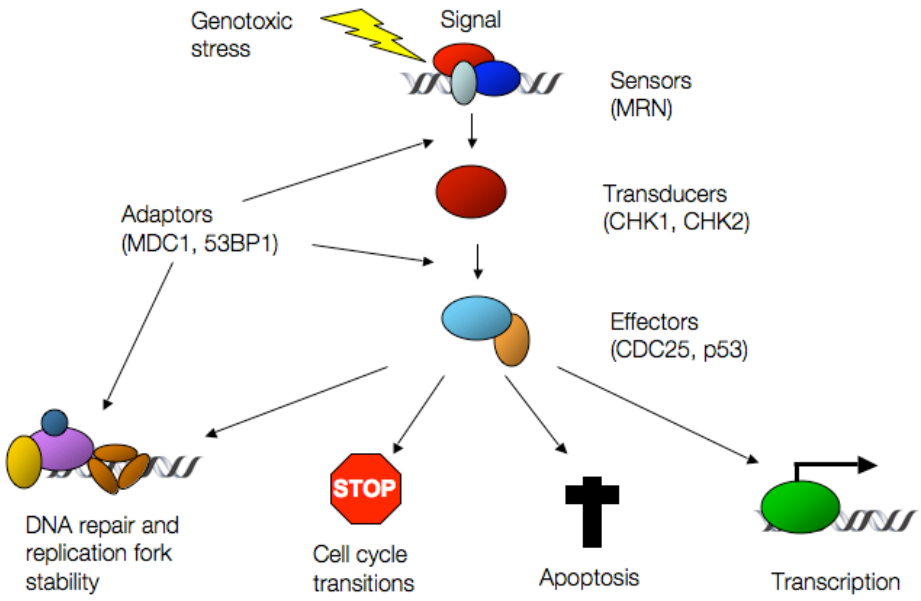

Figure 1.2 A simplified model of the DNA damage response with emphasis on its first signalling events. Examples for the four classes of proteins involved in the DDR are given in brackets (adapted from Zhou and Elledge, 2000).

1.3 DNA double-strand break signalling

DNA double strand breaks (DSBs) in human cells arise either from IR, or from exposure of cells to chemical clastogens. Alternatively, DSBs may be generated by DNA replication over a single-strand break. Inappropriately repaired DSBs can lead to chromosomal aberrations such as translocations. On the other hand, naturally occurring DSBs play an important role in meiotic recombination and are required for V(D)J recombination in developing lymphocytes.

1.3.1 Ionizing radiation-induced nuclear foci formation

Eukaryotic cells response to DSBs by triggering a global DDR. Induction of the signalling network leads to local alteration of the chromatin organisation in the vicinity of the breaks. Moreover, many proteins become recruited into chromatin regions flanking the lesions. These protein aggregates are a hallmark of DSB signalling and are the so-called ionizing radiation-induced foci (IRIF; Shiloh, 2003). They can be visualized as discernible speckles by immunofluorescence and light microscopy. In mammalian cells, one focus reflects protein accumulation at a single DSB.

The assembly of the proteins into IRIF occurs in a highly ordered and strictly hierarchical fashion (reviewed in Bekker-Jensen and Mailand, 2010). One of the early steps in DSB signalling is the activation of the PIKK kinases, ATM, ATR and DNA-PK that seem to regulate many processes of the DDR. ATM exists in an inactive dimer in unstressed cells, but upon IR

its recruitment to DSBs and probably other not yet identified processes or factors trigger the autophosphorylation on Ser1981, resulting in dissociation of the dimer into active monomers (Figure 1.3 A; Bakkenist and Kastan, 2003). ATR is activated upon UV treatment or by long ssDNA stretches that occur upon replication stress.

Among the various targets of the PIKKs is H2AX, a histone H2A variant, making up about 10 – 15 % of total H2A in the nucleosomes of a cell. Its sequence is almost identical with the sequence of H2A with the exception of an 11 amino acid long extension at the very C-terminus containing Ser139 that is phosphorylated upon DSBs formation (Rogakou et al., 1998). Kinetic measurements revealed that the phosphorylation occurs within minutes and reaches a maximum level about 30 min post IR. Phosphorylated H2AX (γH2AX) was proposed to cover a large chromatin region flanking the DSBs, but the modification does not spread on neighbouring undamaged chromosomes (Rogakou et al., 1999). H2AX is phosphorylated by ATM and in some cases DNA-PK in response to DSBs, and by ATR after UV exposure or replicative stress.

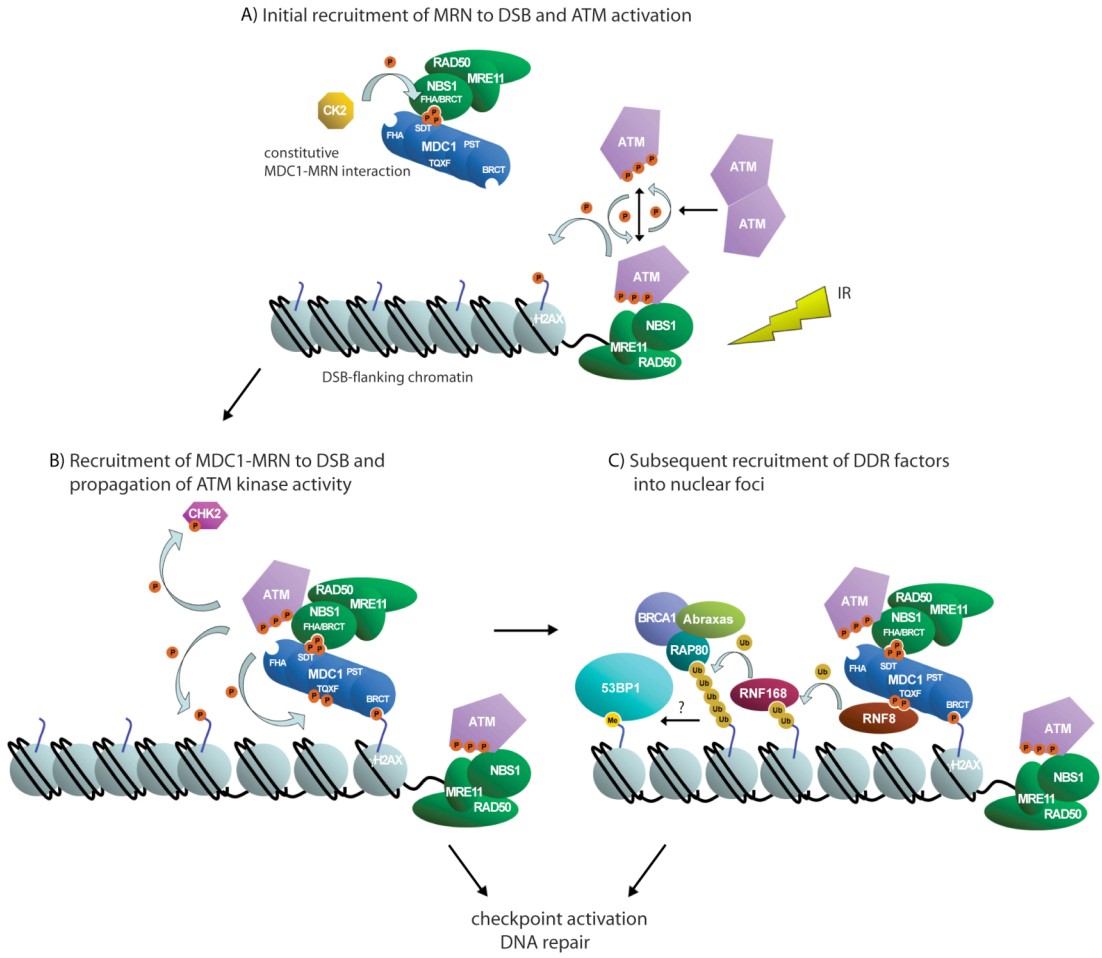

Figure 1.3 The current model of the assembly of proteins into IRIF. See text for more details (Jungmichel and Stucki, 2010).

With its C-terminal BRCT tandem domain, the mediator of checkpoint protein 1 (MDC1) directly binds to γH2AX (Stucki et al., 2005). MDC1 is a large protein of about 2000 aa, harbouring different domains involved in various processes (reviewed in Jungmichel and Stucki, 2010). Since it directly interacts with γH2AX, it is essential for the retention of most proteins in IRIF and it is the main organizer of the chromatin domains adjacent to DSBs. Via an internal region containing several SDT motifs that are phosphorylated by casein kinase 2 (CK2) on both serine and threonine, it recruits the MRE11/RAD50/NBS1 (MRN) complex to the γH2AX-enriched chromatin domain (Figure 1.3 B). The function of the MDC1-dependent chromatin retention of the MRN complex is not yet known.

The discovery of an additional phosphorylation site at the C-terminus of H2AX added a new layer of complexity to the regulation of γH2AX. The last amino acid of H2AX, Tyr142, is phosphorylated constitutively in unstressed cells by the WSTF kinase, a component of the WICH chromatin-remodelling complex (Xiao et al., 2009). Upon DNA damage, this residue is dephosphorylated by the EYA phosphatase. Doubly phosphorylated H2AX does not bind MDC1, but binds JNK1 kinase that is initiating apoptosis (Cook et al., 2009). It was proposed that these mechanisms are engaged in decision making between repair and apoptosis, although the exact impact of one phosphorylation status on the other is still elusive (Stucki, 2009).

MDC1 is phosphorylated by ATM at specific TQXF motifs (Kolas et al., 2007; Mailand et al., 2007; Matsuoka et al., 2007). Via the FHA domain, RNF8 directly binds to these phosphorylated threonines and is hence recruited to the γH2AX-enriched chromatin regions (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang et Elledge, 2007). RNF8 is an E3 ubiquitin ligase and initiates a complex and tightly regulated ubiquitylation cascade that generates binding sites for downstream factors with mechanisms that are not fully understood so far. The identity of the substrates of RNF8 is not yet fully discovered, but several lines of evidence suggest H2A and H2AX to be ubiquitylated by RNF8. To add to the complexity, two additional E3 ubiquitin ligases seem to be essential for the formation of the K63-linked polyubiquitin chains in damaged chromatin. RNF168 encompasses two motifs interacting with ubiquitin (MIUs) that are responsible for its targeting to sites of damage via the interaction with ubiquitylated H2A and H2AX (Doil et al., 2009; Stewart et al., 2009). Its recruitment is strictly RNF8-dependent. Both RNF8 and RNF168 interact with UBC13, the only E2 ubiquitin-conjugating enzyme known to exclusively catalyze the formation of K63-linked polyubiquitin chains (Huen et al., 2008). Interaction with another E3 ligase, HERC2, mediates the preferential association of RNF8 with UBC13 (Bekker-Jensen et al., 2010). HERC2 is phosphorylated by ATM, which leads to the interaction with RNF8 via the FHA domain. Di- or oligomerisation allows RNF8 to be recruited to MDC1 via the FHA domain of one RNF8 molecule while interacting with HERC2 with the FHA domain of the other molecule of the dimer.

All these ubiquitylation steps are required for BRCA1 retention in damaged chromatin (Figure 1.3 C). The so-called BRCA1 A complex is recruited due to its component RAP80, a protein that binds via two ubiquitin-interacting motifs (UIMs) to ubiquitylated histones (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007; Yan et al., 2007; Wu et al., 2009). The function of the other components of the complex (BRCC36, Abraxas, BRE and MERIT40), are not yet identified (Huen and Chen, 2010). The core component, Abraxas, remains stably expressed throughout the cell cycle in contrast to BRCA1 that displays low protein levels during G1 and might have additional functions besides recruiting BRCA1 to damaged chromatin (Choudhury et al., 2004).

The chromatin accumulation and retention of 53BP1 at sites of DSBs is also dependent on the above described ubiquitylation events, although neither direct interaction between 53BP1 and ubiquitylated H2AX or H2A was observed nor any bridging factors comparable with the BRCA1 A complex was found for 53BP1. Moreover, 53BP1 harbours a tandem tudor domain that can interact with methylated histones and is essential for the retention of 53BP1 at damaged chromatin (Huyen et al., 2004). Structural analysis of the domain displayed specific affinity for di-methylated forms of H4K20 (Botuyan et al., 2006). This chromatin mark is locally increased in the chromatin regions adjacent to DSBs (Pei et al., 2011). The responsible methyltransferase MMSET was found to interact in a DNA damage-dependent manner with the BRCT domains of MDC1. MDC1 is proposed to dimerise and thereby to enable the recruitment of MMSET to sites of damage. However, this recently found mechanism does not reveal the exact involvement of the ubiquitylation events in the recruitment and retention of 53BP1.

The establishment of such IRIF is even more complex, since the roles of deubiquitylating enzymes and the sumoylation machinery in the regulation of the accumulation of this plethora of proteins are just emerging (Doil et al., 2009; Galanty et al., 2009; Morris et al., 2009).

While the order of recruitment of all these factors is quite well understood, the functional implication of this massive accumulation of proteins in chromatin regions spanning over megabases of DNA is still unknown.

1.4 The DNA damage checkpoints

To ensure the propagation of accurate copies of the genome to the subsequent generation, cells can activate several cell cycle checkpoints upon DNA damage. These are responsible for arrest or delay of cell cycle progression that provides time for DNA repair. The three DNA damage checkpoints are presented in the next chapters.

1.4.1 The G1/S checkpoint

To prevent replication of damaged DNA in S phase, cells exposed to damaging agents in G1 phase activate the kinase ATM. This assures then in a multistep process cell cycle arrest in G1 by activating the two different branches of the G1/S checkpoint (Figure 1.4). A direct substrate of ATM is the checkpoint kinase CHK2. This then directly phosphorylate CDC25A on multiple serine residues enhancing its ubiquitylation and hence proteasomal degradation (Sorensen et al., 2003; Zhao et al., 2002a). CDC25A is the activating phosphatase of the CDK2/cyclinE and CDK2/cyclinA complex, the cell cycle regulating kinases for the G1/S transition. CDC25A removes an inhibitory phosphate on CDK2. The inhibition of CDK2 blocks replication firing by preventing loading of Cdc45 onto the pre-replication initiation complex at origins, hence polymerase α that is required to prime the replication cannot be loaded. Degradation of CDC25A occurs very rapidly since it is only dependent on posttranslational modifications but it is also very transient and this pathway is thus not capable to arrest the cell cycle permanently in G1.
However, a second pathway exists that is dependent on transcription. Upon DNA damage in G1, the tumour suppressor p53 is phosphorylated by both ATM/ATR and the transducer kinases CHK1/CHK2. Additionally, the E3-ubiquitin ligase Mdm2 that normally binds p53 is also phosphorylated by ATM/ATR (Khosravi et al., 1999; Maya et al., 2001). Both these mechanisms lead to stabilisation of protein levels and increased transcriptional activity of p53. P53 is involved in the transcription of many genes, the most prominent for the G1/S checkpoint is the CDK inhibitor p21. Its accumulation during this phase of the cell cycle blocks progression into S phase.

1.4.2 The Intra-S-phase checkpoint

In the S phase of the cell cycle, human cells replicate their entire genome of around three billion basepairs to obtain two identical copies – one for each daughter cell. In order to preserve the integrity of the genome the duplication has to occur error-free, which is especially demanding if the DNA is damaged during S phase. Cells that experience genotoxic stress during S phase slow the replication process down to allow the cells to repair the lesions before finishing replication. If the damage cannot be repaired during this transient delay, cells complete replication and exit S phase, but arrest in G2 phase to deal with the DNA damage.
Cells that are irradiated during S phase or encounter DSBs otherwise during DNA replication have evolved a so-called intra-S-phase checkpoint to prevent initiation of new replicons and thereby slowing down DNA replication (reviewed in Bartek et al., 2004). However, this checkpoint seems to have no influence on fork progression, since the rate of DNA synthesis at already fired origins is almost unaltered (Merrick et al., 2004). Triggering of the checkpoint is strongly dependent on the two kinases ATM and ATR. ATM is supposed to be activated

first in still not fully understood mechanisms, but local chromatin modifications, the MRN complex and other factors might influence ATM autophosphorylation on serine 1981 and hence dimer dissociation to fully active monomers. ATM activity is required for the resection of the DNA ends that gives rise to long 3' single-stranded overhangs. These are then coated with the replication protein A (RPA). Long stretches of ssDNA coated with RPA seem to be the trigger for ATR signalling, although precise mechanisms of ATR activation are not yet known (Myers and Cortez, 2006). ATR-ATRIP heterodimers bind to the RPA coated stretches and lead to activation of ATR with involvement of other proteins (Zou et Elledge, 2003). ATR phosphorylates then and activates CHK1, which leads to phosphorylation of CDC25A and induces its proteolysis via the proteasome (Figure 1.4). CDC25A is the activating phosphatase removing inhibitory phosphate groups on cyclinE/CDK2 or cyclinA/CDK2 to promote loading of CDC45 initiation factor to origins and hence firing of origins. Thus, via CDC25A degradation the firing of late origins is prevented. Besides CHK1, CHK2 that is activated by ATM upon DSBs also phosphorylates CDC25A and increases its turnover upon irradiation in concert with CHK1 (Sorensen et al., 2003).

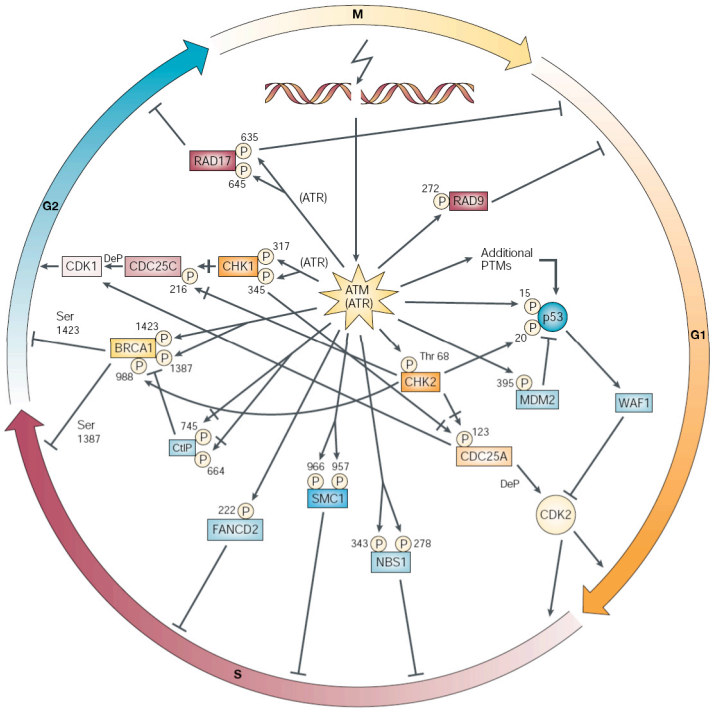

Figure 1.4 The basics of checkpoint signalling at the different cell cycle phases. See text for details (Shiloh, 2003).

Additionally, a second branch of the intra-S-phase checkpoint seems to exist that involves direct ATM phosphorylation of NBS1 and SMC1 (Figure 1.4). However, how phosphorylated SMC1 interferes with DNA replication is still unknown.

Both branches of the intra-S-phase checkpoint lead to slowing down of the overall replication rate in cells exposed to IR. Depletion of a protein involved in either of the two branches leads to a so-called radio-resistent DNA synthesis (RDS) phenotype. Many factors in addition to the above-mentioned appear to be involved in the intra-S phase checkpoint, because depletion of MDC1, 53BP1, FANCD2, BRCA1 and BRCA2 lead to an RDS phenotype. However, their exact role in the intra-S-phase checkpoint remains unknown.

1.4.3 The G2/M checkpoint

Before entering mitosis it is crucial for the cells that their genomic material is completely and properly replicated and the two sister chromatids untangled. There are two mechanisms that prevent cells to enter mitosis with damaged chromosomes. If cells encounter damage during S phase, they tend to arrest in G2 phase to have time to repair the damage before entering mitosis. This accumulation in G2 is more pronounced in cells defective in the intra-S-phase checkpoint and is not dependent on the kinase ATM (Xu et al., 2002). However, cells that encounter DNA damage during G2 phase of the cell cycle are transiently arrested in an ATM-dependent manner. Neither of the two checkpoints depends on the other (Xu et al., 2002). To measure the latter checkpoint, mitotic cells are normally counted by fluorescence-activated cell sorting (FACS) after a one-hour recovery time post IR, to assure that only cells that have been in the G2 phase upon treatment are counted (Xu et al., 2001).

Similar to the G1 checkpoint, the key targets of the transient G2/M checkpoint are the phosphatases CDC25 (Figure 1.4). They remove the inhibitory phosphate of the cell cycle kinase CDK1/cyclinB, which regulates most processes in mitosis. ATM/ATR phosphorylate and thus activate CHK1/CHK2 and these kinases then inhibit the CDC25 phosphatase by sequestering them in subcellular compartments or by initiating their degradation. However, other upstream regulators of CDC25 or CDK1/cyclinB, such as Polo-like kinases PLK1 and PLK3 are also involved in the G2/M checkpoint (Nyberg et al., 2002).

As in the G1 cell cycle checkpoint, p53 is stabilised and activates transcription of several cell cycle inhibitors such as p21. In addition, the expression of GADD45 and 14-3-3σ is also induced and required for efficient arrest (Taylor and Stark, 2001). Interestingly, cells that are deficient for p53 can still arrest in G2, indicating that additional mechanisms may cooperate with p53 in this checkpoint. The mediator proteins 53BP1 and BRCA1 are also involved in the G2/M checkpoint but the mechanisms by which they regulate this checkpoint remain unknown so far (Xu et al., 2001; Wang et al., 2002; DiTullio et al., 2002; Fernandes-Capetillo et al., 2002). In addition, cells from mice lacking H2AX manifest a G2/M checkpoint defect after exposure to low, but not high, doses of IR (Fernandes-Capetillo et al., 2002).

1.5 DNA double-strand break repair

Two major repair pathways evolved to deal with DSBs. Non-homologous end joining (NHEJ) rejoins two dsDNA ends together. Small deletions of some nucleotides can occur and therefore, NHEJ is called error-prone. Homologous recombination (HR) on the other hand is an error-free repair pathway, but it requires a DNA template with homology to the damaged region. The sister chromatid is the best template since it is identical to the damaged chromosomes, but it only exists during S and G2 phases of the cell cycle. If the homologous chromosome is used for repair, HR leads to loss of heterozygosity.

The choice between these repair pathways depends on the cell cycle stage. Studies of either HR- or NHEJ-deficient cells revealed that these two pathways compete for the repair of DSBs (Delacote et al., 2002). NHEJ-deficient cells show reduced repair efficiency in any cell cycle stage, whereas HR-deficient cells have a significant DSB repair defect only during the S/G2/M phases (Rothkamm et al., 2003; Hinz et al., 2005). The mechanisms by which cells choose between theses two repair pathways has been a matter of investigation for the past few years. Growing evidence arises that for efficient HR CDK activity is required. First, resection, the key step of HR, is dependent on CDK activity (Jazayeri et al., 2006). Second, several proteins essential for resection were found to be phosphorylated by CDKs. The orthologue of CtIP in budding yeast, Sae2, is phosphorylated by Cdk1p and this phosphorylation is critically required for resection (Huertas et al, 2008). In mammalian cells, phosphorylation of a putative CDK site in CtIP leads to its interaction with the BRCT domains of BRCA1 and is required for the resection of DSBs and for the repair via HR (Yu and Chen, 2004; Yun and Hiom, 2009). These findings indicate that the regulation of the initiation of the HR repair pathway occurs at the level of BRCA1 and CtIP through CDK activity (reviewed in Branzei and Foiani, 2008). However, it is likely that other mechanisms contribute to the choice between repair pathways in DSB repair.

1.5.1 Non-homologous end joining

The major pathway to repair DSBs in mammalian cells is NHEJ. This repair mechanism leads to a simple rejoining of two ends of dsDNA. Nevertheless, it is critical that the right ends are joined, otherwise NHEJ is dangerous because it can lead to chromosomal translocations. Thus, both termini of a DSB must be held in close proximity, most likely by protein-protein interactions between DNA end-binding factors. In addition, some small deletions of DNA sequence can occur and thus, this repair pathway is error-prone.

Significant structural diversity of the DSB lesions exists, which is dependent on how the DSBs are initiated. Flaps, small ssDNA overhangs or even hairpins may form at the break site and demand a great mechanistic flexibility. Directly at the breaks, the Ku complex, a heterodimer of the proteins Ku70 and Ku86, binds to the duplex DNA ends (Figure 1.5;

Mimori and Hardin, 1986; Blier et al., 1993). Its X-ray structure reveals a ring-like shape with positively charged amino acids pointing inwards that engage in unspecific interactions with the backbone of duplex DNA molecules (Walker et al., 2001). The Ku complex is proposed to change its conformation upon binding to DNA, because only when bound to DNA, it interacts with DNA-PK catalytical subunit (DNA-PKcs; Yaneva et al., 1993; Lieber 2010). Among the various substrates of DNA-PKcs the NHEJ factors XRCC4 and Artemis are listed but also DNA-PK itself. The autophosphorylation inhibits DNA-PK activity and induces dissociation of the DNA-PK complex (Chan and Lees-Miller, 1996; Merkle et al., 2002).

The Ku proteins bound to DNA together with DNA-PKcs serve also as a recruiting platform for nucleases, polymerases, and ligases. They are not recruited sequentially but in a manner independent of each other, resulting in diversity of the outcomes of joining events. Nucleases cleave off some nucleotides or open hairpins, DNA polymerases can add some nucleotides in a template-independent manner and ligases seal any two dsDNA or ssDNA together that are in close proximity in a sequence independent manner.

Evidence exists for several of such factors to play a role in NHEJ. Artemis is a nuclease with single-stranded 5'-3' exonuclease activity. Upon its phosphorylation by DNA-PKcs it can also acquire endonuclease activity specific for hairpins and ssDNA overhangs (Ma et al., 2002). It is dispensable for the repair of 'clean' DSBs that are induced by restriction enzymes or etoposide and that do not have damaged nucleotides at the ends (Zhang et al., 2004; Riballo et al., 2004). However, the repair of at least ten percent of DSBs induced by IR is dependent on Artemis (Riballo et al., 2004).

The 5' DNA kinase and 3' DNA phosphatase activity of polynucleotide kinase (PNK) is required for processing DNA ends during NHEJ. The ends of the broken DNA might not have a 5' phosphate group, which is required for ligation by the ligase complex. Such ends can be 'taken care of' by PNK and converted into substrates for the ligase complex (Karimi-Busheri et al, 1999; Chappell et al., 2002).

Polymerases μ and λ can both interact with the Ku proteins bound to DNA via their BRCT domain and might be responsible for template-independent DNA synthesis during NHEJ (Ma et al., 2004). Additionally, polymerase activity of pol λ is also required to fill in gaps arisen from ligation of complementary ssDNA (Lee et al., 2004).

The ligase complex, DNA ligase IV with XLF and XRCC4, ligates the DNA ends. This complex shows a great flexibility and is able to ligate across gaps and incompatible ends (Gu et al., 2007a; Gu et al., 2007b). It is found in a complex with DNA-PKcs and its recruitment is dependent on DNA-PKcs (Chen et al., 2000; Calsou et al., 2003).

Alternative NHEJ acts when factors of the classical NHEJ pathway such as Ku, XRCC4 or DNA ligase IV are absent. These repair events entail frequently small deletions and require short stretches of homology between the joinable DNA ends. Microhomology-mediated end-joining (MMEJ) is the most important alternative pathway. This repair mechanism involves modest resection of DNA of less than 100 nucleotides until regions of homology are exposed

and lead to reattachment of the two DNA ends of the break. The DNA is processed further by nucleases that cut off flaps and overhangs and by DNA polymerases to fill in the gaps.

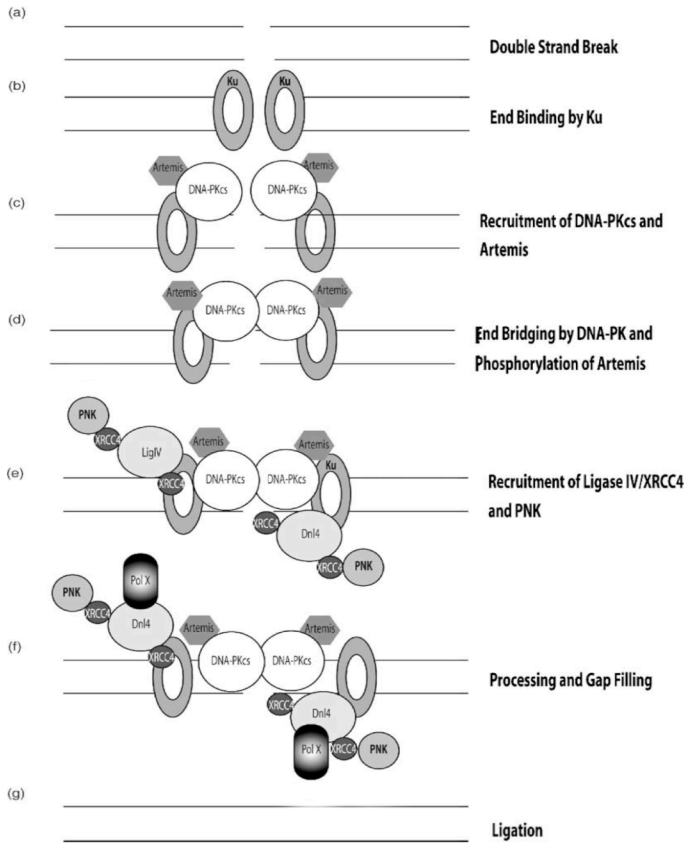

Figure 1.5 A model for mammalian NHEJ. DSBs are first bound by the Ku complex. Interaction with DNA-PKcs leads to recruitment of several additional factors that perform smaller deletions, addition of some nucleotides, and at the end ligation of DNA ends or sealing of the nicks occur (adapted from Hefferin and Tomkinson, 2005).

1.5.2 Homologous recombination

The repair of DSBs via homologous recombination is error-free but it requires the presence of identical or similar DNA sequences that are not always present in mammalian cells. NHEJ is therefore more frequently used to repair DSBs in mammalian cells, but there are some damages that can only be repaired via HR, such as collapsed replication forks or breaks induced by inhibiting topoisomerase I by camptothecin (CPT).

A critical step for the initiation of HR is the resection of dsDNA ends to generate 3' single-stranded overhangs. Both CtIP and the MRN complex are required for the initiation of resection, but the processive 5'→3' exonucleolytic degradation is thought to be carried-out by other enzymes (Sartori et al., 2007; Jazayeri et al., 2006). Possible candidates are the exonuclease EXO1 and the helicase BLM together with the nuclease DNA2. Both seem to

act in resection, in a partially redundant manner (Figure 1.6). CtIP was recently observed to restrain the exonuclease activity of EXO1 *in vitro* and might regulate the processivity of EXO1 *in vivo* (Eid et al., 2010).

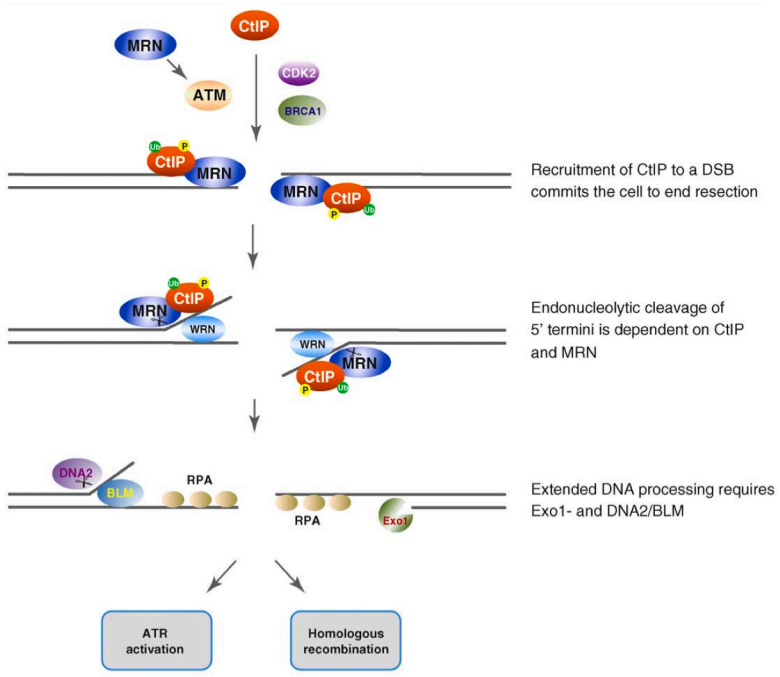

Figure 1.6 A model for resection. MRN and CtIP are required for the initiation of resection. Processivity of resection is performed by EXO1 or by BLM together with DNA2 (You and Bailis, 2010).

The exposed 3' overhang ssDNA is coated with the replication protein A (RPA) that melts the secondary structure of the DNA. On one hand, this long stretches of RPA-coated DNA can activate ATR and hence initiate a global DDR, and on the other hand, it is required for DNA strand invasion and homology search. The nucleoprotein filaments are formed by replacing RPA with the ssDNA-binding protein RAD51 in a mechanism that requires the mediators BRCA1/BARD1 and BRCA2/DSS1 (Figure 1.7). The direct loading of RAD51 is performed by BRCA2, which is shown to interact directly with RAD51 (Sharan et al., 1997; Wong et al., 1997; Pellegrini et al., 2002). These filaments invade then duplex DNA, of the sister chromatid at homologous sequences. Once found, the invading strand sets up a D-loop intermediate. It pairs with the complementary strand of the homologous DNA region and displaces the other. The 3' overhang can then prime the DNA synthesis using the sister chromatid as a template. Two models exist for the resolution of this intermediate. First, the double strand break repair (DSBR) model suggests that the D-loop captures the other end of the break and forms a Holliday junction (HJ; Figure 1.7 pathway D and E). HJs are then resolved either by a complex containing the helicase BLM and Topoisomerase IIIα to non-crossover, or by MUS81-EME1 to form crossover products (Wu and Hickson, 2003; Chen et al., 2001a; Constantinou et al., 2002). However, more HJ resolvases were identified recently

in higher eukaryotes, such as GEN1 and SLX4, which might refine the resolution of the HJ (Ip et al., 2008; Svendsen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009). Crossovers are mainly produced during meiotic recombination and are very rare in somatic cells. Thus, in somatic cells, the D-loop intermediate is thought to be mainly resolved by synthesis-dependent strand annealing (SDSA). This model proposes minimal migration of the D-loop and pairing of the invading and elongated strand with the second end of the break (Figure 1.7 pathway C).

In absence of homologous sequences, single-strand annealing can occur. When repetitive sequences are exposed by resection of the DNA the two strands of either side of the break can anneal to each other (Figure 1.7 pathway F). DNA flaps are then removed by a nuclease and nicks are sealed by DNA synthesis and ligation. One-ended DSBs that can arise from uncapped telomeres or collapsed replication forks after replication over a nicked region are repaired by BIR (break-induced repair). The invading strand copies the DNA until the end of the chromosome (Figure 1.7 pathway G). Therefore, this repair mechanism is prone to loss of heterozygosity.

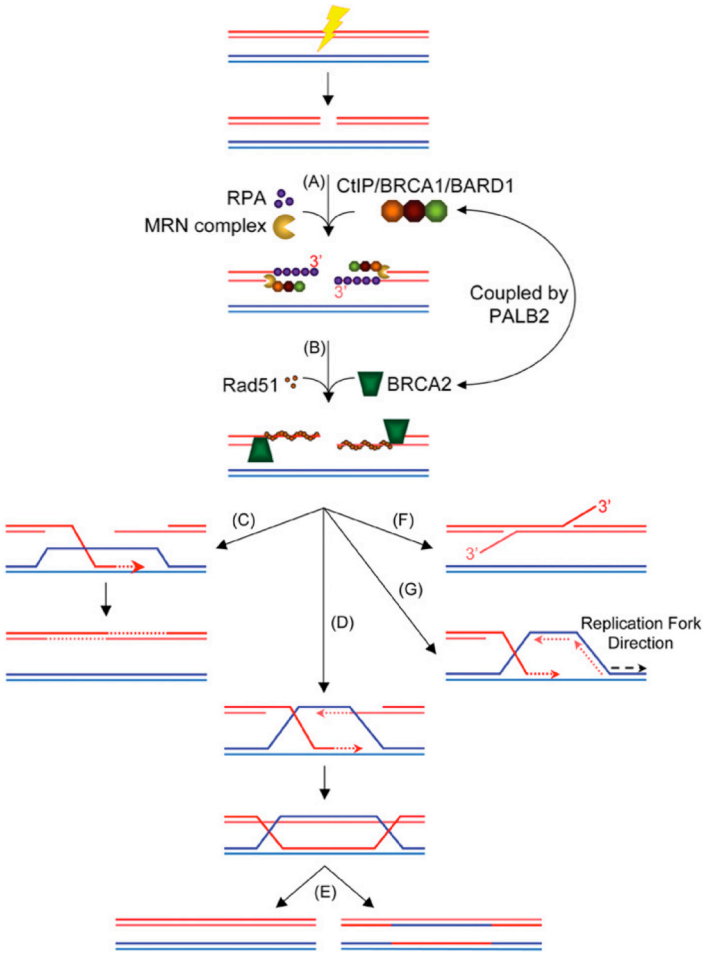

Figure 1.7 A model for mammalian HR with an emphasis on the various ways of the resolution of the D-loop intermediate (Hartlerode and Scully, 2009).

1.6 Chromatin remodelling complexes involved in the DDR

DNA is wrapped around nucleosomes that fold the genome into highly condensed chromatin. To establish and maintain chromatin structure, enzymes that can modify, move or exchange nucleosomes are required. These enzymes deposit nucleosomes during replication, slide nucleosomes along the DNA double helix to expose regulatory elements of transcription or remove them to open access for RNA polymerases during transcription. In the DDR, they open up chromatin to allow binding of repair enzymes and are involved in the resection to generate long stretches of ssDNA during homologous recombination.

Some chromatin modifying enzymes have a DNA-dependent ATPase domain. These are the ATP-dependent chromatin remodelers. They display high affinity towards nucleosomes and are commonly found in bigger complexes with several subunits. According to their unique domains (besides the ATPase domain) they can be classified into four classes.

The SWI/SNF family was first discovered in yeast. With their bromodomains these family members can interact with acetylated lysines, which leads to increased remodelling efficiency (Ferreira et al., 2007). These remodelers are implicated in various processes. Their involvement in DNA repair became evident when the yeast RSC complex was observed to be recruited to damaged chromatin and to interact with Ku and MRE11. It was reported to play a role in DSB repair in both NHEJ and HR (Shim et al., 2005; Chai et al., 2005).

Extensive studies performed in yeast revealed the involvement of the INO80 family members in DNA repair. INO80 null mutants display hypersensitivity to DNA-damaging agents (Shen et al., 2000; Van Attikum et al., 2004; Morrison et al., 2004). Consequently, INO80-containing protein complexes were found in chromatin regions adjacent to the breaks, as detected by chromatin immunoprecipitation. In addition, INO80 helps to expose DNA to the resection machinery during HR, as INO80 mutants fail to create 3' overhangs. Moreover, INO80 is required for checkpoint activation in yeast (Van Attikum et al., 2004; Tsukuda et al., 2005; Van Attikum et al., 2007). These chromatin remodelers often have orthologues in higher eukaryotes, yet the involvement of these in the DDR has still to be elucidated.

Recently, a member of the fourth family, the chromo-ATPase/helicase-DNA-binding remodelers, was shown to play a role in genome stability (Larsen et al., 2010; Smeenk et al., 2010; Polo et al., 2010). CHD4 was found to be enriched in chromatin upon irradiation of cells and to be recruited to sites of DNA damage (Larsen et al., 2010). Knock down of CHD4 in human cells leads to several DDR phenotypes: decreased survival upon IR, deregulation of the cell cycle and reduced efficiency of DNA repair.

Thus, chromatin remodelers emerge as important genome caretakers that facilitate checkpoint signalling and DNA repair.

2 The FHA/BRCT region of NBS1 and its role in the DDR

2.1 Introduction

2.1.1 Mediators/adaptors of the DDR

Mediators (also called adaptors) form a relatively new class of proteins involved in the DDR. They show no enzymatic activity, but they are nevertheless important for specific DDR processes by e.g. recruiting certain DDR proteins to the site of damage, by organizing multi-protein complexes, or by facilitating the interaction between enzymes and their substrates. Most of these mediators act downstream of the PIKK kinases in the DDR and are thought to be important for amplifying and fine-tuning the responses. Known representatives of mediators in the DDR are MDC1, 53BP1, NBS1, Claspin, Brit1/Mcph1, and BRCA1 (reviewed in Harper and Elledge, 2007).

MDC1 for example is a large adaptor protein of about 2000 aa length and consists of at least five distinct regions or domains. Each of these domains has been implicated in a specific DDR-related function. The main mode of action of MDC1 is to undergo specific protein-protein interaction with other DDR proteins and thus, to recruit and organise protein complexes in DNA damage-induced nuclear foci (reviewed in Jungmichel and Stucki, 2010).

Mediators usually contain several protein-protein interaction domains. For example, MDC1 contains the protein-protein interaction modules forkhead associated (FHA) and the BRCA1 C-terminal (BRCT) domains, which are described in the next section.

2.1.2 FHA and BRCT domains

The initiation and regulation of the DDR is critically depended on posttranslational modifications, mostly on Ser/Thr phosphorylations of the signalling and effector proteins. Some of these phosphorylations are recognized by specific phosphorylation-dependent protein-protein interaction modules. Such modules are the FHA domain and BRCT domain (reviewed in Mohammad and Yaffe, 2009).

FHA domains were first discovered by bioinformatics studies of the forkhead transcription factor family (Hofmann and Bucher, 1995). They are found in hundreds of transcription factors as well as rather frequently in DDR factors. To date, about 20 of FHA domain structures have been solved by either nuclear magnetic resonance (NMR) or X-ray crystallography. Though their sequences show low homology, their fold is characteristic for FHA domains. Eleven beta-strands fold into two beta-sheets, with loops and turns that connect the beta-strands and that are responsible for the ligand-selectivity (Figure 2.1). They exclusively bind phosphothreonines, but show very diverse specificity for sequence context (Durocher et al., 2000). Most of the FHA domains select for the amino acid at the plus three

position (pTXXD or pTXX(I/L/V); Durocher and Jackson, 2002). But there are other FHA domains that recognize residues N-terminal to the pThr, bind to TQ clusters or interact with an extended binding surface besides contacting the highly conserved pThr (Bernstein et al., 2005; Li et al., 2004; Mahajan et al., 2008).

Well-characterised FHA domains are the ones of the checkpoint kinases CHK2 and Rad53, its homologue in yeast. It is activated by DNA damage via phosphorylation by ATM or ATR at its SQ/TQ-rich cluster in its N-terminus and then it transduces the signal further downstream by phosphorylating effector proteins. Phosphorylation at threonine 68 of CHK2 leads to the interaction with an FHA domain of another CHK2 molecule and hence to its dimerisation and oligomerisation (Ahn et al., 2002, Li et al., 2008). However, FHA domains are not always in involved in protein dimerisation/multimerisation. More frequently, they interact with residues on other proteins. For example the ubiquitin ligase RNF8 interacts via its FHA domain with MDC1 that is phosphorylated by ATM on threonines in the TQXF motifs.

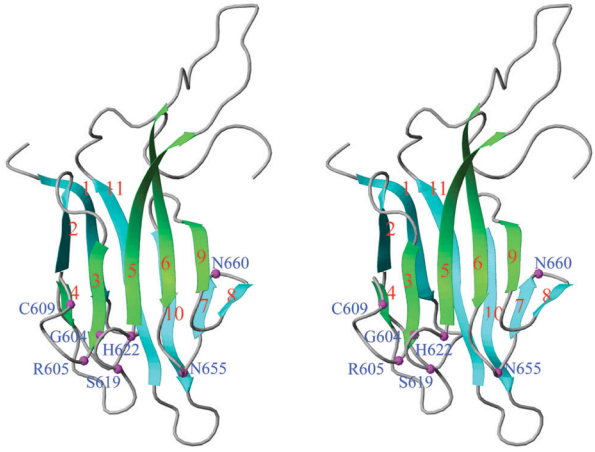

Figure 2.1 Basic structural features of the FHA domain. Stereoview of the FHA2 domain of the S. cerevisiae kinase Rad53. The large β sheets are colored in green and blue, respectively, and the conserved residues are highlighted with a purple label (Mahajan et al., 2008).

The first BRCT domain was described in BRCA1 (Bork et al., 1997). BRCT domains are less abundant than FHA domains and appear only in proteins connected with DNA damage signalling or repair so far. Not every BRCT domain is involved in phospho-specific interactions. So far, all of the BRCT domains that mediate phospho-specific interactions have been found to occur in tandem pairs. The more N-terminal BRCT domain stabilises the phosphorylated residue, whereas the cleft between the two BRCT domains forms a hydrophobic pocket that engages in interactions with the aromatic residues at the plus three position after the phosphorylation site (Figure 2.2; Clapperton et al., 2004; Shiozaki et al., 2004). BRCT domains generally bind stronger to phosphoserine than to phosphothreonine (Manke et al., 2003; Stucki et al., 2005). BRCT tandem domains that display phospho-specific interaction are found for instance in BRCA1 and MDC1. BRCA1 interacts with at

least three different phosphoproteins, giving rise to three distinct protein complexes, each of which with its specific role in the DDR. The tandem BRCT domain of MDC1 interacts with γH2AX initiating and maintaining the accumulation of proteins along the chromatin domain adjacent to the DSB (Stucki et al., 2005). In contrast to BRCA1, MDC1 binds with its BRCT tandem domain the C-terminus of a protein, stabilising the interaction by contacting the COOH group. Elongation of the C-terminus of H2AX by two amino acid or phosphorylation of the last tyrosine leads to loss of interaction with MDC1 (Stucki et al., 2005; Xiao et al., 2009).

A few proteins contain only a single BRCT domain, such as XRCC1, REV1, pol λ and PARP family members, others, such as TopBP1 and PTIP, contain multiple BRCT domains. The exact role of each of these BRCT domains has not yet been elucidated.

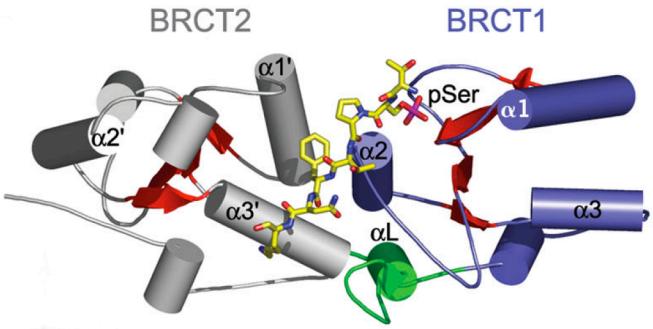

Figure 2.2 The fold of the BRCT tandem domain of BRCA1 with its bound phospho-peptide. The first BRCT1 domain is colored in blue, the second fold in grey. The linker alpha helix is colored in green (Clapperton et al., 2004).

Two proteins are known that harbour both FHA and BRCT tandem phospho-specific interaction modules and in one of them they are located adjacent to each other: NBS1, the adaptor protein of the MRN complex, has a conserved FHA domain at its N-terminus, followed by a BRCT tandem domain (Becker et al., 2006; Xu et al., 2008). Interestingly, there is no spacer between the two domains, indicating that they may form one single structural domain. NBS1 was shown to interact with phosphorylated MDC1 via its N-terminus, but the involvement of these phospho-specific interaction modules still needed to be elucidated (Chapman & Jackson, 2008; Melander et al, 2008; Spycher et al, 2008; Wu et al., 2008).

2.1.3 The MRN Complex

The composition and architecture of the MRN complex

The function of the MRN complex in the DDR is multifaceted. It acts as a sensor, that recognises DSBs, but it also controls signalling and exercises the role of an effector protein. Supporting their prominent function in the DDR, the two MRN complex components MRE11

and RAD50 are highly conserved across species and homologues are found in bacteria, archaea and throughout the eukarya (de Jager et al., 2004; Hopfner et al., 2000; You et al., 2005). NBS1 is less conserved and is only found in eukaryotes.

The overall structural architecture of the MRN complex is mostly determined by the structure of RAD50 (Figure 2.3 A). It can be separated into distinct 'head', 'coil' and 'hook' domains. RAD50 contains globular ABC-ATPase domains at both ends of the protein that form a bipartite ATP-binding cassette. These domains are connected via an anti-parallel coiled-coil region with a Zn-hook domain in its middle (Figure 2.3 B). The hook domain can either interact intramolecularly with the hook domain of the other RAD50 molecule of the same complex or intermolecularly with the hook domain of a RAD50 molecule of another MRN complex.

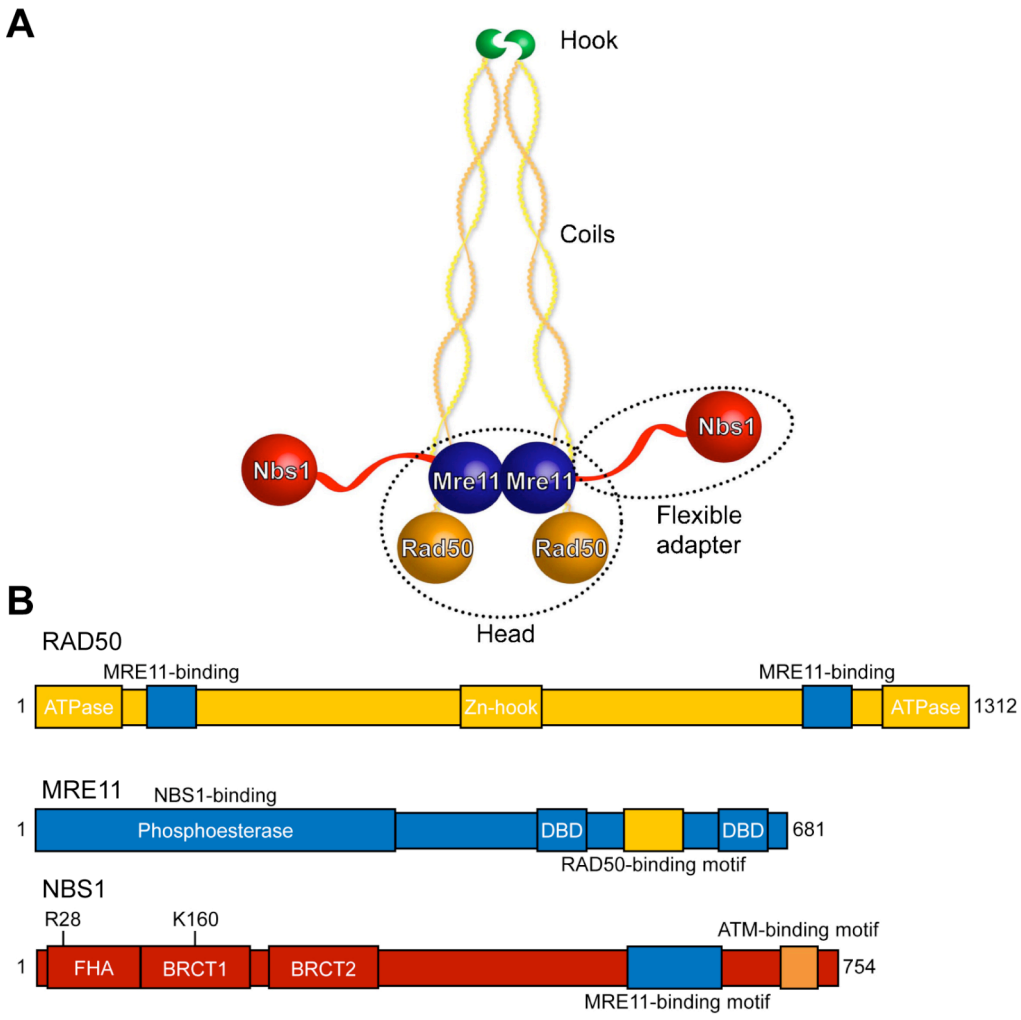

Figure 2.3 The architecture of the MRN complex. A) MRE11 together with the ATPase domain of RAD50 forms the 'head' domain, the coiled-coil domain of RAD50 links the two RAD50 molecules via the 'hook' domain or two RAD50 molecules of two independent MRN complexes, and the adapter NBS1 is flexible connected via its unfolded C-terminus (Williams et al., 2010). B) A schematic view of the architecture of the three subunits. Relevant domains are depicted.

Together with the globular ATPase domains of two RAD50 molecules, a MRE11 dimer forms the globular head domain of the MRN complex. This domain is mainly responsible for the DNA-binding activity of the MRN complex, either via the ATPase domain of RAD50 or via the C-terminal DNA binding domains of MRE11.
Flexibly attached to the head domain is the NBS1 protein via its C-terminal MRE11 interaction motif (Desai-Mehta et al., 2001; Tauchi et al., 2001; You et al., 2005). At its N-terminus it harbours a rigid globular domain consisting of the two phospho-specific interaction modules, the FHA domain and a BRCT tandem domain. Its C-terminal half is most likely not folded and interacts with MRE11 and ATM (Desai-Mehta et al., 2001; Falck et al., 2005; You et al., 2005).

Structural analysis and biochemical *in vitro* studies showed that the MRN complex shows DNA tethering properties. It is thought to hold the two ends of the DSB in close proximity to avoid dissociation of the DNA ends and thus, improper joining events (de Jager et al., 2001; Chen et al., 2001b; Hopfner et al., 2002; Moreno-Herrero et al., 2005). The core domain of the MRN complex associates with termini of linear dsDNA, whereas the coiled-coil region of RAD50 forms flexible arms of about 500 Å that search for other termini of DNA by interaction with other RAD50 molecules via the hook domain. In line with this, mutation of the Zn-binding site of the RAD50 hook domain disrupts the DNA end tethering function (Hopfner et al., 2002).
MRE11 has a well-characterised endo- and exonuclease activity. The endonuclease activity is essential for initiation of HR (Buis et al., 2008; Williams et al., 2008) and the exonuclease activity is required for MMEJ (Rahal et al., 2010; Zhuang et al., 2009). The ATPase activity of RAD50 enhances the MRE11 endonuclease activities (Paull and Gellert, 1999; Trujillo and Sung, 2001).
NBS1 lacks any enzymatic activity, but it is nevertheless important for the complex. If any of the three components are depleted, the cells show lower protein levels of the two other subunits. Additionally, NBS1 is responsible for the nuclear localisation of the whole complex and upon mutation of the MRE11 interaction motif of NBS1, the MR complex is localised to the cytoplasm and degraded (Desai-Mehta et al., 2001; Tauchi et al., 2001).

All three subunits of the MRN complex are essential proteins. Knock out mice that have lost any of the three MRN subunits are embryonic lethal (Luo et L., 1999; Xiao and Weaver, 1997; Zhu et al., 2001). Inherited mutations in their genes lead to human autosomal recessive disorders. NBS1 is mutated in patients suffering form Nijmegen breakage syndrome (NBS; Carney et al., 1998). These patients show microcephaly, mental retardation, deformation of cranial-facial bones, immunodeficiency, and predisposition to malignancy (Young and Painter, 1989). Mutations in MRE11 are responsible for ataxia-telangiectasia-like disorder (ATLD), which lead to ataxia and neurodegeneration similar to AT patients (Stewart et al.,

1999). Recently, a Rad50 mutation was detected in a patient with NBS-like symptoms (Waltes et al., 2009).

Cells of these patients or cells derived from animal models show radiosensitivity, checkpoint defects, and display increased amounts of chromosomal aberrations (Kang et al., 2002; Bender et al., 2002; Williams et al., 2002; Luo et al., 1999). These phenotypes demonstrate the versatile function of the MRN complex in the DDR.

The function of the MRN complex in the context of the cellular response to DSBs

First of all, the MRN complex is involved in the initiation of DSB signalling. It acts as a sensor protein and rapidly binds to DSBs via its 'head' domain containing the MRE11 nuclease and the ATPase domain of RAD50. In an MRN-dependent manner via multiple protein-protein interactions, ATM is recruited to the DSB and activated in a mechanism that is not yet fully understood. It is clear though that this mechanism involves the MRN complex (Lee and Paull, 2004; Lee and Paull, 2005). The MRN complex increases the concentration of ATM at the site of the break and promotes the autophosphorylation of ATM that is critical for its activation. Especially at low doses of irradiation it seems to be required for the efficient activation of ATM (Horejsi et al., 2003). Based on the fact that the MRN complex is involved in sensing DSBs and initiating a signalling response, it is clear that cells depleted of either of the subunits suffer from checkpoint defects.

Activated ATM leads to phosphorylation of H2AX and to the accumulation of a large amount of protein complexes to the sites of damage. Via direct interaction between the adaptor subunit NBS1 with phosphorylated residues on MDC1, the whole MRN complex is also retained in the γH2AX chromatin domain that is established over megabases adjacent to the break (Maser et al., 1997). The precise function of these protein agglomerations has yet to be established, and the functional role of the MRN complex in these γH2AX chromatin domains is also not yet known.

However, the MRN complex is not only involved in DNA damage signalling and cell cycle checkpoint events, but it is also functionally implicated in both of the two major DSB repair pathways. HR is initiated by resection of the DNA ends to give rise to kilobase-seized stretches of 3' ssDNA overhangs. MRE11 does not display 5'→3' exonuclease activity and thus, could not be directly responsible to form these overhangs, but nonetheless it was shown that MRN together with its binding partner CtIP is essential for proper resection (Jazayeri et al., 2006; Sartori et al., 2007).

A role for the MRN complex in classical as well as in the alternative NHEJ pathway has been suggested (Rass et al., 2009; Xie et al., 2009). End-joining efficiency was reduced in MRE11 knock down cells and the resected tracks occurring in the alternative NHEJ pathway were shortened. In summary, the MRN complex is an important DDR factor involved in many different processes of the DSB signalling and repair.

The interaction of the MRN complex with MDC1

The accumulation of the MRN complex in γH2AX-positive damaged chromatin was observed a while ago (Maser et al., 1997). NBS1 was discovered to be the subunit responsible to retain the whole MRN complex in damaged chromatin. Moreover, its N-terminal FHA/BRCT module was implicated in the recruitment of the MRN complex to the γH2AX chromatin domain (Zhao et al., 2002b; Cerosaletti and Concannon, 2003; Horejsi et al., 2004). It was previously suggested that NBS1 directly interacts with γH2AX (Kobayashi et al., 2002). However, evidence arose that there must be an adaptor protein linking this interaction between the MRN complex and γH2AX. First of all, knock down of MDC1 by siRNA prevented MRN complex recruitment and accumulation in IRIF (Goldberg et al., 2003; Lukas et al., 2004; Stewart et al., 2003). Second, mouse embryonic fibroblasts (MEFs) isolated from MDC1 knock out mice show no MRN foci (Lou et al., 2006). Finally, MDC1 was found to bind directly to γH2AX via its C-terminal tandem BRCT domains (see above) (Stucki et al., 2005).

Recently, our lab and others identified a region in MDC1 that acts as an NBS1-interaction site, the so-called SDT motifs (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). The SDT repeat region is characterized by conserved patches of 8-10 amino acids comprising serine and threonine residues typically separated by an aspartate and further embedded in an acidic sequence environment. This SDT region interacts with the MRN complex in a phosphorylation-dependent manner. In human MDC1, six SDT motifs were identified and deletion of at least five of them leads to complete abrogation of MRN IRIF formation (Melander et al, 2008; Spycher et al, 2008). Analysis of NBS1 recruitment to sites of DSBs revealed that upon expression of an MDC1 version lacking the SDT regions, NBS1 only accumulates in micro-IRIF but is not found in the broader chromatin compartments usually covered by γH2AX and MDC1 (Chapman and Jackson, 2008).

Interestingly, MDC1 and MRN exist in a complex even in undamaged cells. This interaction is dependent on the activity of the acidophilic casein kinase 2 (CK2) for which the SDT motifs form consensus phosphorylation sites (Spycher et al, 2008; Wu et al, 2008). Both serine and threonine residues in each SDT motif are phosphorylated by CK2 *in vivo* and only doubly phosphorylated pSDpT motifs are capable to mediate the interaction with NBS1 (Melander et al, 2008; Spycher et al, 2008). NBS1 harbours two phospho-specific interaction modules at its N-terminus. The exact role of these domains and their functional implication upon interaction with MDC1 was the subject of this project.

2.2 Results

2.2.1 Both FHA domain and BRCT domains of NBS1 interact with MDC1 *in vitro*.

Until recently, sequence comparison and structure predication algorithms indicated that the N-terminal region of NBS1 contained an FHA domain and one single BRCT domain (reviewed in D'Amours & Jackson, 2002). Four years later, a second putative BRCT domain at the N-terminus of NBS was discovered by means of a refined bioinformatic analysis (Becker et al, 2006). The existence of two BRCT domains downstream of the FHA domain at the NBS1 N-terminus was partially confirmed by a recently published NMR structure of the second (more C-terminal) BRCT domain (Xu et al., 2008). Interestingly, there seems to be no spacer between the FHA domain and the putative tandem BRCT domain, indicating that these domains may form one single compact globular structure. Moreover, the conservation of key phospho-binding amino acid residues in the BRCT tandem domain suggests that like the FHA domain, it may act as a phospho-specific protein-protein interaction module.

Several groups have recently shown that the FHA domain of NBS1 directly associates with a constitutively phosphorylated region in MDC1, the SDT repeat region (Chapman & Jackson, 2008; Melander et al, 2008; Spycher et al, 2008). Mammalian MDC1 contains a total of six SDT motifs, and at least three of these are required for efficient MRN accumulation at sites of DSBs (Spycher et al, 2008). This may indicate that more than one binding site with affinity to the phosphorylated SDT region may exist in NBS1. Thus, we decided to test whether the intact NBS1 BRCT tandem domain was required for the efficient association of NBS1 with the full-length phosphorylated SDT region. To this end, we phosphorylated (or mock treated) the human GST-tagged MDC1 SDT fragment and assessed its ability to interact with *in vitro*-translated full-length NBS1 protein that carried point mutations in key residues within its phospho-binding FHA and BRCT tandem domains, respectively. As shown before, full-length wild type NBS1 efficiently interacted with the phosphorylated SDT region of MDC1 (Figure 2.4 A; Melander et al, 2008; Spycher et al, 2008). Interestingly, FHA domain single mutant (R28A) and a BRCT tandem domain single mutant (K160M) also showed residual SDT binding activity. However, a double phosphopeptide-binding mutant (R28A/K160M), failed to bind to the phosphorylated SDT region (Figure 2.4 A). This indicates that both the FHA domain and the BRCT tandem domain are capable to interact with the phosphorylated MDC1 SDT region *in vitro*.

NBS1 does not exist on its own in the nuclei of mammalian cells, as it is always associated with MRE11 and RAD50. Thus, our assay conditions with the *in vitro*-translated NBS1 do not very well reflect a physiological situation where NBS1 is part of a heterotrimeric complex. Therefore, we co-expressed all three subunits of the MRN complex in insect cells and tested their binding affinity to the phosphorylated SDT region of MDC1. Also in the context of the

intact MRN complex, wild type NBS1 efficiently bound to the phosphorylated SDT region (Figure 2.4 B). Surprisingly though, neither the FHA mutant (R28A) nor the BRCT tandem domain mutant (K160M) was capable to associate with the phosphorylated SDT region (Figure 2.4 B). This indicates that when NBS1 exists in a heterotrimeric complex with MRE11 and RAD50, both the intact FHA domain and the BRCT tandem domain of NBS1 are essential for efficient association with phosphorylated MDC1. It is currently not clear why the NBS1 single mutants still interacted with the phosphorylated SDT region when *in vitro*-translated, while in the context of the heterotrimeric MRN complex, they did not. But it is possible that when NBS1 is an integral part of the MRN complex, its N-terminal phosphopeptide binding region may be sterically less accessible so that efficient association with the SDT region is only possible when both FHA domain and BRCT tandem domain are contributing to the interaction.

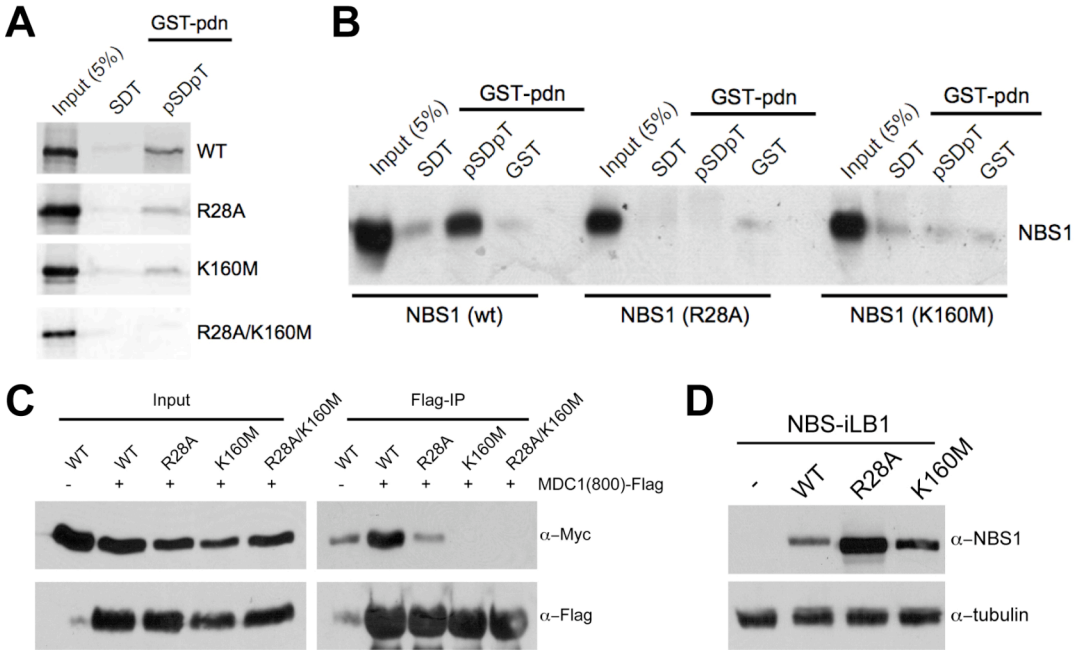

Figure 2.4 Both the FHA domain and the tandem BRCT domain of NBS1 are required for the interaction with the phosphorylated SDT region of MDC1 *in vitro*. (A) Purified MDC1 GST-SDT fragment was pre-incubated with CK2 and ATP. The fragment was then incubated with in vitro-translated 35S-labeled NBS1 wild type or mutants for 1 h, washed and analyzed by SDS-PAGE and autoradiography. (B) Purified MDC1 GST-SDT fragment was pre-incubated with CK2 and ATP. The fragment was incubated with purified MRN complex where the NBS1 subunit was either wild type or contained a point mutation in the FHA domain (R28A) or in the BRCT tandem domain (K160M). Bound proteins were separated on SDS PAGE followed by immunoblotting. The blots were probed with a polyclonal antibody against NBS1. (C) HEK 293T cells were transiently transfected with Flag-tagged MDC1(1-800) fragment and Myc-tagged NBS1 wild type and mutants as indicated. Flag beads were used for co-immunoprecipitation and Myc antibody for Western blot analysis. (D) NBS1 protein levels of NBS-iLB1 fibroblasts stably transduced with wild type, R28A or K160M mutant NBS1 were analyzed by SDS PAGE followed by immunoblotting.

Since an intact NBS1 FHA domain and BRCT tandem domain appear to be essential for the interaction with the MDC1 SDT region, we next asked if both of these domains were also involved in complex formation with MDC1 in mammalian cell extracts. Therefore, we co-expressed a Flag-tagged 800 amino acid N-terminal fragment of MDC1 (containing the SDT region) with Myc-tagged full-length NBS1 wild type and mutant derivative, respectively and tested their association by co-immunoprecipitation. Significantly, only wild type NBS1 interacted with the MDC1 fragment in extracts prepared from the transfected cells, while neither FHA and BRCT tandem domain single-mutants (R28A; K160M), nor the double mutant (R28A/K160M) showed any significant binding activity towards MDC1 (Figure 2.4 C). Hence, both the FHA domain and the BRCT tandem domain are required for efficient binding of NBS1 to the phosphorylated SDT region of MDC1.

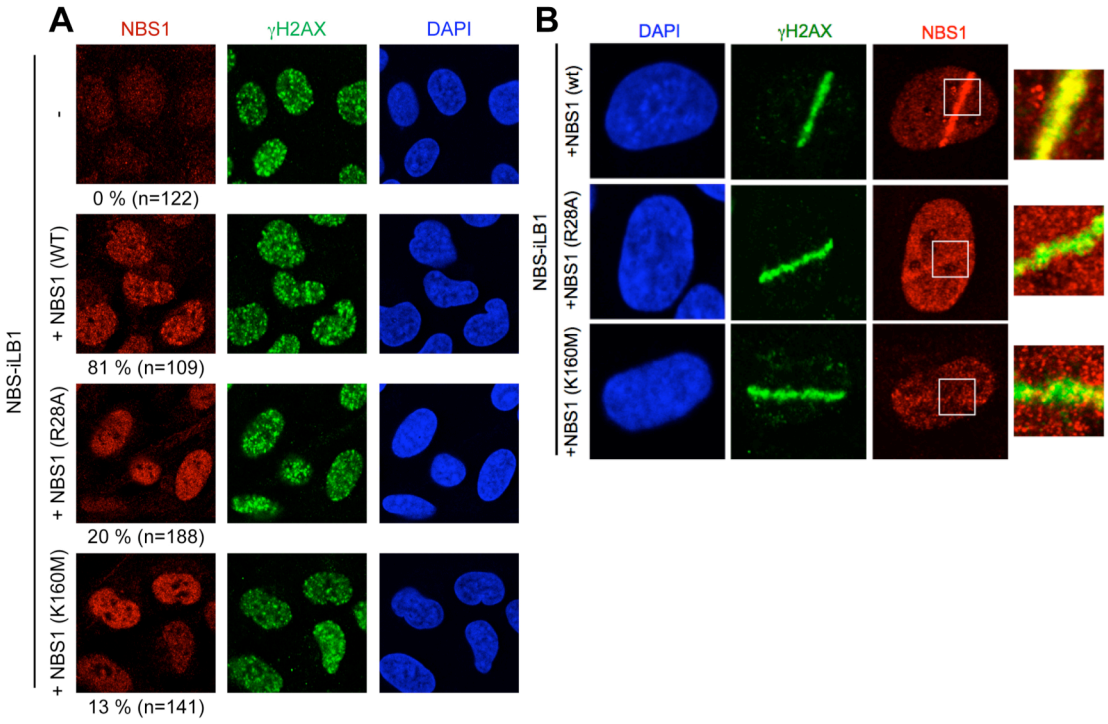

Figure 2.5 The BRCT tandem domain of NBS1 is required for focal accumulation of the MRN complex at sites of DSBs *in vivo*. (A) NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild type NBS1 and R28A and K160M mutant NBS1, respectively, were irradiated with 5 Gy. Irradiated cells were incubated for 1 h, fixed with methanol and probed with the indicated antibodies. Cells were then analyzed by confocal microscopy and nuclear foci positive cells were counted for statistical evaluation. (B) NBS-iLB1 fibroblasts stably transduced with wild type NBS1 and R28A and K160M mutant NBS1, respectively, were microirradiated as described in Material and Methods. Irradiated cells were incubated for 1 h, fixed with methanol and probed with the indicated antibodies. Cells were then analyzed by confocal microscopy.

2.2.2 The BRCT tandem domain of NBS1 is required for retention of the MRN complex at damaged chromatin *in vivo*.

Next, we sought to investigate whether the K160M mutation in the BRCT tandem domain would also compromise the accumulation of the MRN complex at sites of DSBs as observed earlier for the FHA domain mutant R28A (Cerosaletti & Concannon, 2003; Lukas et al, 2004). To this end, we generated NBS-iLB1 fibroblast cell lines stably transduced with wild type and mutant NBS1, respectively (Figure 2.4 D). Then, we assessed nuclear foci formation of NBS1 in these cell lines by immunofluorescence microscopy. In NBS-iLB1 parental fibroblasts, no NBS1 staining was observed (Figure 2.5 A; top row). However, 81% of the cells stably transduced with wild type NBS1 showed accumulation of NBS1 in foci that overlapped with γH2AX one hour after irradiation with 5 Gy. In contrast, only 20% of cells stably transduced with R28A NBS1 and 13% of cells stably transduced with K160M NBS1, revealed a focal NBS1 staining pattern (Figure 2.5 A), thus indicating that sustained interaction of MRN complex with damaged chromatin requires the phosphopeptide-binding capacity of both FHA and tandem BRCT domains of NBS1.

To develop these findings further, we took the well-established approach of UV-laser microirradiation to induce DSBs in subnuclear volumes (Lukas et al, 2004). Under these conditions, wild type NBS1 accumulated throughout the microirradiated nuclear compartments (Figure 2.5 B). However, both the R28A and K160M mutation prevented NBS1 binding to the γH2AX-coated areas except for a small fraction of the protein scattered along the irradiated path (Figure 2.5 B; see enlarged areas). This residual retention of NBS1 is most probably the fraction of NBS1 that interacts not in an MDC1-dependent manner with the DSB-flanking chromatin but by other means with the ssDNA microcompartments (Bekker-Jensen et al., 2006). Thus, we concluded that phospho-specific binding of both the NBS1 FHA domain and BRCT tandem domain to the MDC1 SDT region is essential for efficient accumulation and retention of the MRN complex in damaged nuclear areas.

2.2.3 The activation of the G2/M checkpoint does not require the BRCT domains of NBS1

We previously proposed that MDC1-mediated accumulation of the MRN complex in chromatin regions flanking DSBs was required for the efficient activation of the G2/M DNA damage checkpoint. This was based on the observation that point mutations in the FHA domain that disrupt its phospho-specific binding, display partial G2/M checkpoint defects both in human and mouse cells (Difilippantonio et al, 2005; Difilippantonio et al, 2007; Spycher et al, 2008). If this interpretation was correct, we would predict that the K160M mutation in the NBS1 BRCT tandem domain also leads to a G2/M checkpoint defect similar to the R28A FHA mutation because MDC1-binding and chromatin accumulation is as

severely compromised in the K160M mutant as it is in the R28A mutant (see above). Surprisingly though, we found that several independent clones of NBS fibroblasts stably transduced with K160M NBS1 activated the G2/M checkpoint almost as efficiently as wild type NBS1 (Figure 2.6 A and B). This indicates that MDC1-binding and MDC1-mediated accumulation of the MRN complex at sites of DSBs is not required for the activation of the G2/M checkpoint.

2.2.4 Experimental uncoupling of the MRN complex from damaged chromatin does not trigger a G2/M checkpoint defect

In order to experimentally verify the above conclusion we exploited an earlier observation that overexpression of a C-terminal fragment of MDC1 comprising its γH2AX-binding C-terminal BRCT domains yielded a strong dominant-negative effect on the accumulation and retention of the DDR proteins at sites of DSBs (Stucki et al, 2005). We reasoned that if our conclusion was correct, we should indeed not observe a G2/M checkpoint defect upon overexpression of the MDC1 BRCT domains. To test this, we used a U2OS cell line carrying a stably integrated, tetracycline regulated, expression cassette directing the expression of the MDC1 tandem BRCT domain fused to yellow fluorescent protein (YFP). As observed before (Stucki et al, 2005), induction of YFP-BRCT expression by the tetracycline analogue doxocycline (DOX) completely abrogated MRN accumulation at sites of DSBs as reflected by both NBS1 foci formation (Figure 2.7 A) and UV-laser microirradiation (Figure 2.7 B). However, induction of YFP-BRCT expression did not trigger a measurable G2/M checkpoint defect after 1 and 3 Gy of IR, respectively (Figure 2.7 C). Significantly though, downregulation of endogenous MDC1 in these cell line still yielded a significant G2/M checkpoint defect, irrespective of whether YFP-BRCT expression was induced or not, thus supporting the previous observation that MDC1 is required for G2/M checkpoint activation (Lou et al, 2003; Lou et al, 2006; Stewart et al, 2003). These data thus support our conclusion that MDC1-mediated accumulation and retention of the MRN complex at sites of DSBs is not required for activation and/or maintenance of the G2/M checkpoint response.

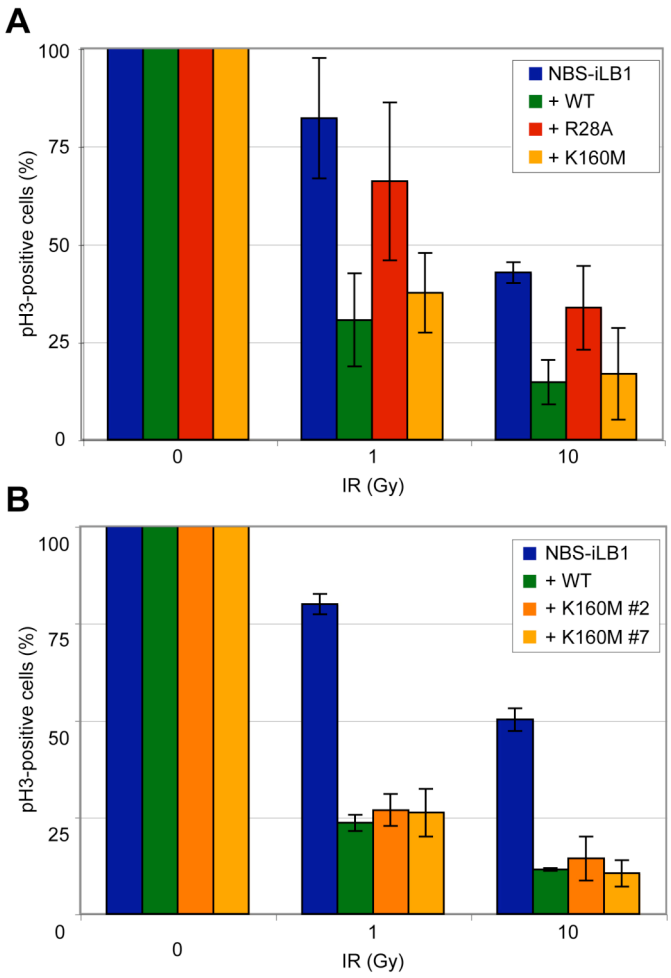

Figure 2.6 Mutation in the tandem BRCT domain of NBS1 does not yield a G2/M DNA damage checkpoint defect. (A) NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild type, R28A, K160M mutant NBS1, respectively, were left untreated or irradiated at 1 and 10 Gy. Cells were harvested 1 h after irradiation, fixed with ethanol and stained with an antibody against phosphorylated H3 (pH3) and propidium iodide. The percentage of pH3-positive cells was determined by fluorescence-activated cell sorting analysis. In this graph, three independent experiments (each performed in triplicate) are summarized. The error bars represent the standard deviation. (B) Same treatment as in (A) of two independent clones of NBS-iLB1 fibroblasts stably transduced with K160M mutant NBS1. NBS-iLB1 parental cells and NBS1-iLB1 cells stably transduced with wild type NBS1 served as negative and positive controls, respectively. Error bars represent standard deviation of triplicates of one experiment.

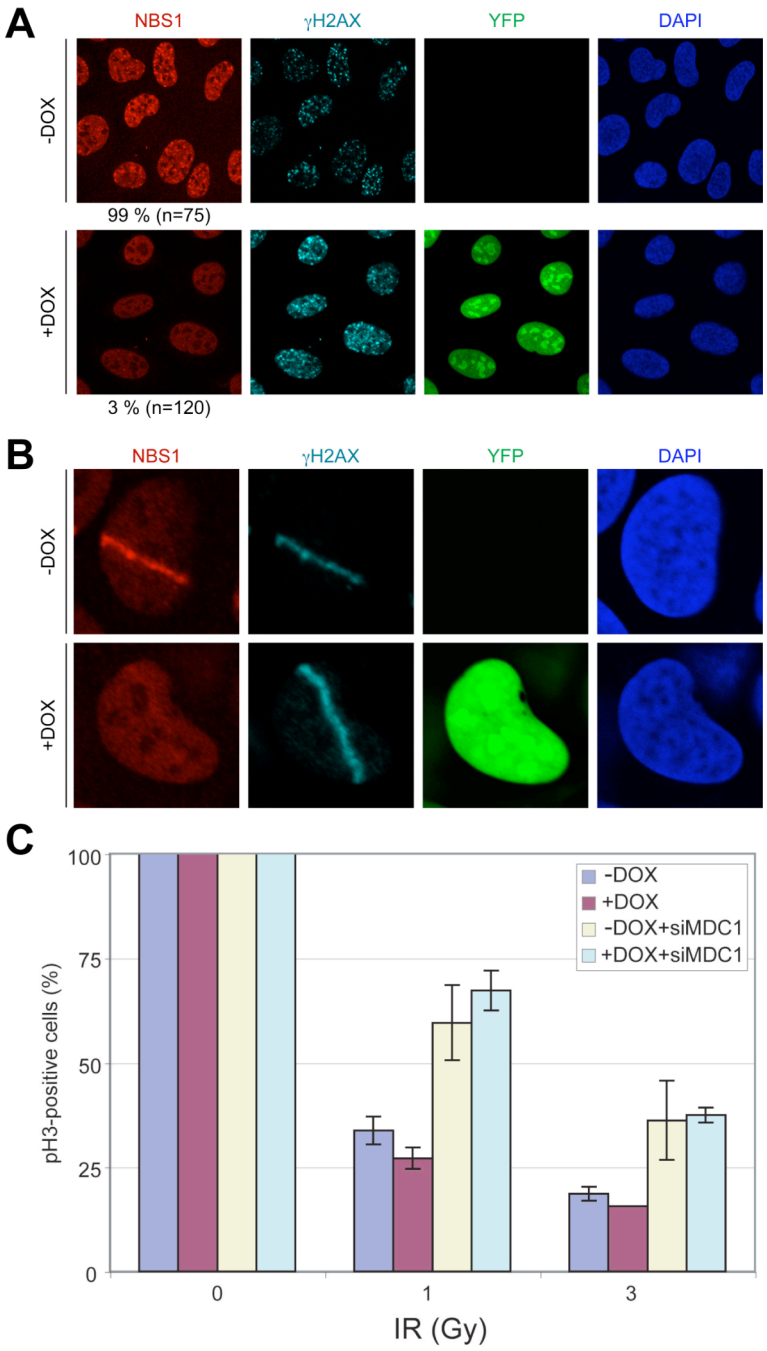

Figure 2.7 Experimental uncoupling of the MRN complex from damaged chromatin does not trigger a G2/M checkpoint defect. (A) Nuclear foci formation of NBS1 in inducible U2OS YFP-BRCT-overexpressing cells after irradiation with 5 Gy. Noninduced cells (top) and YFP-BRCT-expressing cells (bottom). (B) Microlaser-induced DNA damage recruitment analysis of NBS1 in inducible U2OS YFP-BRCT-overexpressing cells. Noninduced cells (top) and YFP-BRCT-expressing cells (bottom). (C) As a control cells were transfected 72 h prior to IR with siRNA against MDC1. Expression of YFP-BRCT fusion protein was induced 8 h before irradiation (+DOX). Mock-induced cells served as control (–DOX). Error bars represent standard deviation of triplicates.

2.2.5 Survival upon IR does not require the FHA domain nor the BRCT domains of NBS1

The separation of function of the FHA and the BRCT domains of NBS1 in the G2/M checkpoint raised the question if these domains are also uncoupled in other functions of NBS1. Moreover, point mutations of these domains are a handy tool to study whether or not the retention of NBS1 is required for a specific function of NBS1, since when only one of both mutants show defects in a certain functional assay, it can be assumed that the interaction with MDC1 and hence its accumulation at sites of DSBs is not required for that specific function.

NBS patient cell lines show reduced overall survival upon IR. The question whether the FHA domain and the BRCT domains are required for survival upon IR was already addressed by several other research groups, but to a very controversial outcome. Furthermore, it was proposed that neither an intra-S-phase checkpoint nor a transient G2/M checkpoint defect would lead to radiosensitivity (Xu et al., 2002). Therefore, we hypothesize that though the FHA domain of NBS1 is required for proper G2/M checkpoint activation it might not be required for survival upon IR. Unless the interaction with MDC1 is required for the survival upon IR, an FHA domain mutant of NBS1 would not be more sensitive to IR than wild type NBS1.

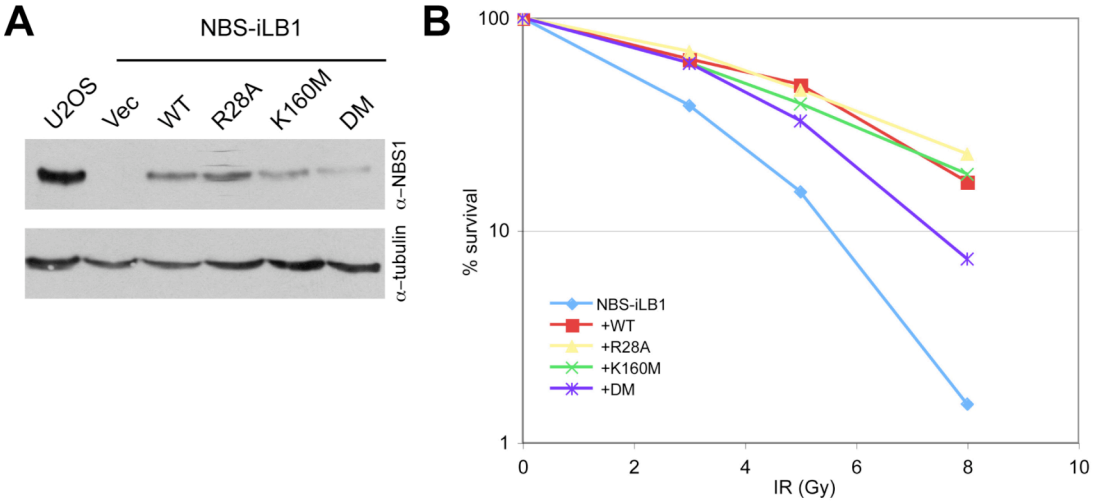

Figure 2.8 NBS-iLB1 cell lines reconstituted with wild type NBS1 or either FHA or BRCT domain mutants rescue the radiosensitivity phenotype of cells lacking full-length NBS1. A) NBS-iLB1 cell lines were stably infected with either wild type NBS1 or the FHA domain mutant (R28A), the BRCT domain mutant (K160M) or the double mutant (R28A/K160M). Cell extracts were prepared and analyzed by SDS PAGE and Western blotting. B) Cell lines of (A) were seeded in low concentration into 6 well plates in triplicates, irradiated with the indicated doses of IR the next day and incubated for additional 8 days. Grown colonies were fixed with MeOH, stained with crystal violet and counted by eyes. Shown is one out of three independent experiments.

To investigate if the retention of NBS1 is required for survival upon IR, we tested reconstituted NBS-iLB1 patient cell lines in a radiosensitivity assay. Since the expression levels of the reconstituted NBS-iLB1 cell lines were highly variable (Figure 2.4 D), new complemented cell lines were created. NBS-iLB1 cells were stably infected with the pLPCX retrovirus harbouring the NBS1 wild type, the FHA domain mutant (R28A), the BRCT domains mutant (K160M) or the double mutant (R28A/K160M). The selected clones expressed NBS1 at similar levels, as analyzed by SDS PAGE and Western blotting (Figure 2.8 A). These cells were then seeded at low density, exposed 16 hours later to sublethal doses of IR and left for 9 days in the incubator. Colonies were stained with crystal violet and counted by eye. NBS-iLB1 cells that do not express full-length NBS1 were more sensitive to IR than the cells reconstituted with wild type NBS1 (Figure 2.8 B). In our hands, both the FHA domain and BRCT tandem domain mutants showed no radiosensitivity and were completely comparable in survival rate with wild type NBS1 cells (Figure 2.8 B). Only the FHA/BRCT double mutant cells showed reduced survival even though not as low as the uncomplemented NBS-iLB1 cell lines. Since the retention of NBS1 is already completely abolished in either of the two single mutants (Figure 2.5), the survival defects of the double mutant cannot be explained by impaired interaction with MDC1; rather, there was only a partial reconstitution of the NBS-iLB1 cells achieved with the double mutant NBS1.

2.2.6 BRCA1 and Artemis are potential interaction partners of the FHA domain of NBS1

Since the FHA domain but not the BRCT tandem domain of NBS1 is involved in the transient G2/M checkpoint, we reasoned that the FHA domain must also interact with another protein besides MDC1, and that this interaction would then mediate the function of NBS1 in the G2/M checkpoint. To address this question, we chose two different approaches. On one hand, we performed an unbiased protein interaction screen for the FHA domain of NBS1. In parallel, we also took a candidate approach (see chapter 2.2.7).

In collaboration with DualsystemsBiotech, we performed a proteomic screen for new phospho-specific interaction partners of the FHA domain of NBS1. An N-terminal fragment of NBS1 (1-382 aa) containing either wild type or mutant FHA domain (R28A) together with the BRCT tandem domain was cloned into a vector containing a C-terminal triple NLS signal from simian virus large T-antigen. NBS1(1-382)-NLS was cloned by gateway cloning into an inducible mammalian expression vector containing a C-terminal Strep/HA tag. With these constructs, Flp-In T-Rex HEK 293 stable cell lines were established. Both cell lines were either left untreated or were irradiated with 10 Gy, respectively, and strep/HA double affinity purifications were performed of all four conditions. Eluates of the second purification were digested with trypsine. Tryptic peptides were purified by HPLC chromatography and peptide

masses were measured by mass spectrometry (direct LC/MS-MS). Proteins were identified by comparing the experimentally obtained fragment spectra with data from theoretically digested and fragmented protein databases. Background proteins were subtracted from the list of possible interactors by using a false positives protein database.
The bait proteins NBS1(1-382) WT or R28A, respectively were found in all the pulldowns, although to a lesser extent in the pulldowns performed with the FHA mutant cell line (Table 2.1). The positive control MDC1 was co-purified with the NBS1(1-382) WT fragment independently of irradiation as expected but not with the R28A mutant (see above).
Since we were interested in proteins binding to the FHA domain of NBS1 and thereby mediating its G2/M checkpoint function, we argued that candidates for such an interaction partner should be found only in the pulldowns performed of the irradiated cells expressing the NBS1 WT fragment. Selecting such proteins and excluding hits that showed up only with two or less unique peptides the list became shortened to two potential interaction partners.

Swissprot no.	Name	WT, -	WT, IR	R28A, -	R28A, IR
O60934	NBS1	80	93	71	56
Q14676	MDC1	98	81	0	0
P38398	BRCA1	0	7	0	0
Q96SD1	Artemis	1	4	0	0

Table 2.1 Protein and the numbers of unique peptides found by mass spectrometry. Only selected hits are shown with at least three unique peptides.

The best hit with seven unique peptides only in the pulldown of irradiated WT cells was BRCA1 (breast cancer susceptibility gene 1). As the name implies, the BRCA1 gene was discovered to be a tumour suppressor gene. When BRCA1 is mutated it may lead to predisposition to breast cancer. BRCA1 is involved in the maintenance of genomic stability although the mechanisms underlying this function are still unclear.
At the N-terminus of BRCA1 a RING finger domain with E3 ubiquitin ligase activity is located. The complex formation with yet another E3 ligase, BARD1, stimulates its activity, although it is not clear if it is required for the function of BRCA1 in the maintenance of genomic stability.
BRCA1 is known to exist in several distinct complexes that are proposed to be involved in different processes of the DDR. At its very C-terminus, BRCA1 harbours a BRCT tandem domain that undergoes phospho-specific interactions with pS-X-X-F motifs (Manke et al., 2003; Yu et al., 2003). Through mutually exclusive binding of its BRCT domains to different proteins, BRCA1 is found in at least three distinct protein complexes. In the so-called BRCA1 A complex, the interaction partner for the BRCT domains is the adaptor protein Abraxas. Via interaction with the UIM-containing protein Rap80, which binds to K63-linked polyubiquitin chains present on broken chromosomes, Abraxas mediates the localisation

and recruitment of BRCA1 to DSB-flanking chromatin (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). BRCC36, BRE, MERIT40 were identified as additional components of this complex. The whole complex is implicated in G2/M checkpoint control and DNA repair, although the mechanisms by which they carry out these functions are still not known.

In the BRCA1 B complex, the BRCT domains interact with BACH1 in a phosphorylation dependent manner to mediate the S-phase checkpoint (Xu et al., 2001; Yu et al., 2003). Furthermore, in a S/G2 phase-specific manner, CtIP and the MRN complex interact with BRCA1 (Yu and Chen, 2004; Sartori et al., 2007), and BRCA1 is implicated in resection of DSBs occurring in these phases of the cell cycle (Schlegel et al., 2006).

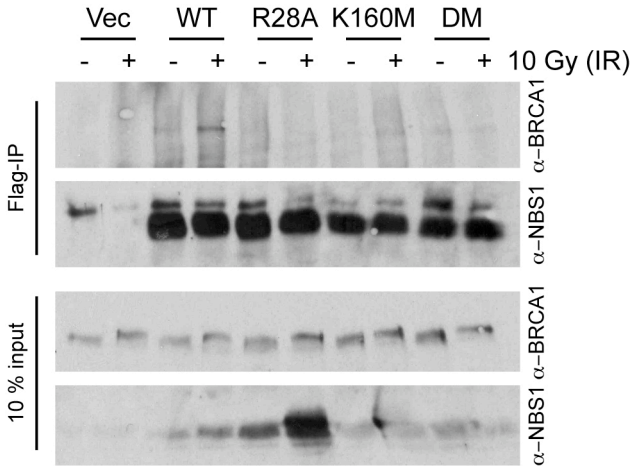

Figure 2.9 BRCA1 interacts with NBS1 only upon irradiation and in a FHA and BRCT tandem domain dependent manner. HEK 293T cells were transfected with NBS1(1-382) WT or mutant constructs and anti-Flag immunoprecipitations were performed. Bound fractions of BRCA1 were analyzed by SDS PAGE and Western blotting.

BRCA1 is involved in transient G2/M checkpoint and it interacts with the MRN complex (Xu et al., 2001; Zhong et al., 1999). Therefore, it would be a good candidate for the missing mediator the G2/M checkpoint function of NBS1's FHA domain.

To verify the data obtained by the screen, we performed an interaction study, where we additionally included the above-described NBS1 BRCT tandem domain mutant (K160M) as well as FHA/BRCT double mutant (R28A/K160M). We transiently transfected HEK 293T cells with a N-terminal fragment (1-382 aa) of NBS1 that was Flag-HA-Strep triple-tagged, containing either wild type, R28A, K160M or R28A/K160M mutant, respectively. The association of this NBS1 fragment with endogenous BRCA1 was analyzed by immunoprecipitation of extracts of untreated and irradiated cells. BRCA1 interacts with the wild-type NBS1 fragment in a damage-dependent manner, but with neither the FHA domain nor BRCT domains mutants of NBS1 (Figure 2.9). Therefore, we concluded that both of these domains are involved in complex formation of BRCA1 and MRN complex, but the association of BRCA1 with the MRN complex is not required for a proper G2/M checkpoint

function. Hence, BRCA1 is most likely not the protein that mediates the G2/M checkpoint function of the FHA domain of NBS1.

The second potential interaction partner for the FHA domain of NBS1 was Artemis. Deficiency of Artemis in humans leads to hypersensitivity to DSB-inducing agents and to the absence of B and T lymphocytes - to the so-called radiosensitive severe combined immune deficiency (RS-SCID). It is a nuclease required for V(D)J recombination that leads to antibody diversity in developing lymphocytes (Moshous et al., 2001). Consistent with the radiosensitivity phenotype of cells derived from such patients, it has been implicated in global DSB repair. It is a single-strand specific 5'→3' exonuclease, but can acquire endonuclease activity specific for hairpins and overhangs upon its phosphorylation by DNA-PKcs (Ma et al., 2002). Moreover, some reports propose that also ATM and ATR can phosphorylate Artemis upon IR or UV, respectively (Zhang et al., 2004). Contradictory results were published concerning Artemis' involvement in NHEJ. Artemis is clearly dispensable for religation of a plasmids digested with restriction enzymes *in vitro* (Zhang et al., 2004). Nonetheless, for complicated breaks where overhangs and hairpins occur, Artemis is suggested to be the responsible nuclease (Budman and Chu, 2005). In line with this, Riballo and colleagues found that directly ligatable etoposide-induced DSBs were repaired efficiently in absence of Artemis. However, the repair of at least 10 % of the DSBs occurring in IR-treated cells is dependent on Artemis as well as on H2AX, 53BP1, MRN and DNA-PK (Riballo et al., 2004). Thus, Artemis seems to be involved in the repair of a subset of DSBs.
The role of Artemis in cell cycle checkpoints is controversial. In one study it was found to be involved in G2/M cell cycle arrest (Zhang et al., 2004), in contrast to another study (Riballo et al., 2004). However, it was never tested if Artemis is involved in the transient G2/M checkpoint in response to IR. Therefore, a potential role of Artemis in the transient G2/M checkpoint has to be described before it may be proposed that it is the interaction partner of the FHA domain of NBS1 that mediates the G2/M checkpoint function of NBS1.

2.2.7 CtIP does not interact in a FHA or BRCT tandem domain dependent manner with NBS1

Our top candidate for an additional interaction partner of the FHA domain of NBS1 was CtIP, the CtBP interacting protein. It was reported that CtIP interacts with the MRN complex, both via its N-terminus and C-terminus with NBS1 (Sartori et al., 2007; Chen et al. 2008). CtIP was implicated in the G2/M checkpoint (Yu and Chen, 2004; Greenberg et al., 2006; Yu et al., 2006). Moreover, in the fission yeast *schizosaccharomyces pombe (sp),* two studies recently showed that spNBS1 interacts via its FHA domain with Ctp1, the yeast orthologue of human CtIP. This interaction was shown to be critical for survival upon treatment with DSBs-inducing agents such as CPT, phleomycin and methyl methanesulfonate (Lloyd et al.,

2009; Williams et al., 2009). These groups identified SXT motifs in Ctp1 that directly interact with the FHA domain of spNBS1. These motifs resemble the SDT motifs in human MDC1. The phosphorylation of the SDT motifs in MDC1 is constitutively performed by CK2. Therefore, we hypothesized that CK2 could be responsible for the phosphorylation of the residues in mammalian CtIP. Hence, we searched in the sequence of human CtIP for the minimal consensus sites of CK2 (S/T-X-X-D/E) and found several possible sites towards the C terminus of CtIP. To investigate if they are indeed phosphorylated by CK2, we performed *in vitro* kinase assays with a recombinantly expressed and purified GST-tagged fragment of CtIP(650-897), containing these sites. Recombinant CK2 was capable to phosphorylate this fragment, but not two other CtIP fragments containing either a part of the N-terminus or the very C-terminus (Figure 2.10 A).

To test if the phosphorylation of this fragment of CtIP would indeed lead to interaction with NBS1, we performed GST pulldown experiments. Prior to the pulldowns, GST-CtIP(650-897) was phosphorylated by CK2 *in vitro* and then incubated with either HeLa nuclear extract or *in vitro*-translated NBS1. Unfortunately, no conclusive results were obtained after several attempts of either experiment, most likely due to the fact that the MRN complex already binds to beads alone and the interaction between CtIP and the MRN complex was very weak (data not shown).

Therefore, we switched to other approaches such as immunoprecipitation to address the question if the interaction between CtIP and NBS1 is dependent on the FHA domain of NBS1. First, HEK 293T cells were co-transfected with Myc-tagged NBS1 WT, FHA or BRCT domain mutants and with full-length Flag-tagged CtIP and co-immunoprecipitations were performed using anti-Flag beads. As a positive control NBS1 WT was co-expressed with Flag-tagged MDC1(1-800). Overexpressed CtIP interacted only very weakly with NBS1 WT and to the same extent also with the FHA and the BRCT domains mutants (Figure 2.10 B).

In order to use more physiological conditions, the reconstituted NBS-iLB1 patient cell lines (see Figure 2.4 D) were used in a co-immunoprecipitation experiment using anti-MRE11 antibodies. The whole MRN complex including the reconstituted NBS1 was immunoprecipitated. Endogenous CtIP was co-immunoprecipitated with MRN complex containing the wild-type NBS1. In contrast, no CtIP was co-immunoprecipitated with MRN complex containing either the FHA or BRCT domain mutants (Figure 2.10 C). However, even in cells lacking full-length NBS1, CtIP co-immunoprecipitated with MR complex. CtIP was reported to be able to interact with all three components of the MRN complex *in vitro* (Yuan and Chen, 2009). Moreover, the NBS cell line we were using still weakly expresses a C-terminal 70 kDa fragment of NBS1. Since NBS1 is interacting with MRE11 via its C-terminus, the p70 fragment also coimmunoprecipitated with MRE11 (Figure 2.10 C). This MR-p70 subcomplex might also provide an interaction platform for CtIP. Overall, it can be concluded that CtIP can interact with an MR complex or at least with an MR complex containing the C-

terminal fragment of NBS1, but also with normal MRN complex. Only when either the FHA or the BRCT domains are mutated, the interaction is lost.

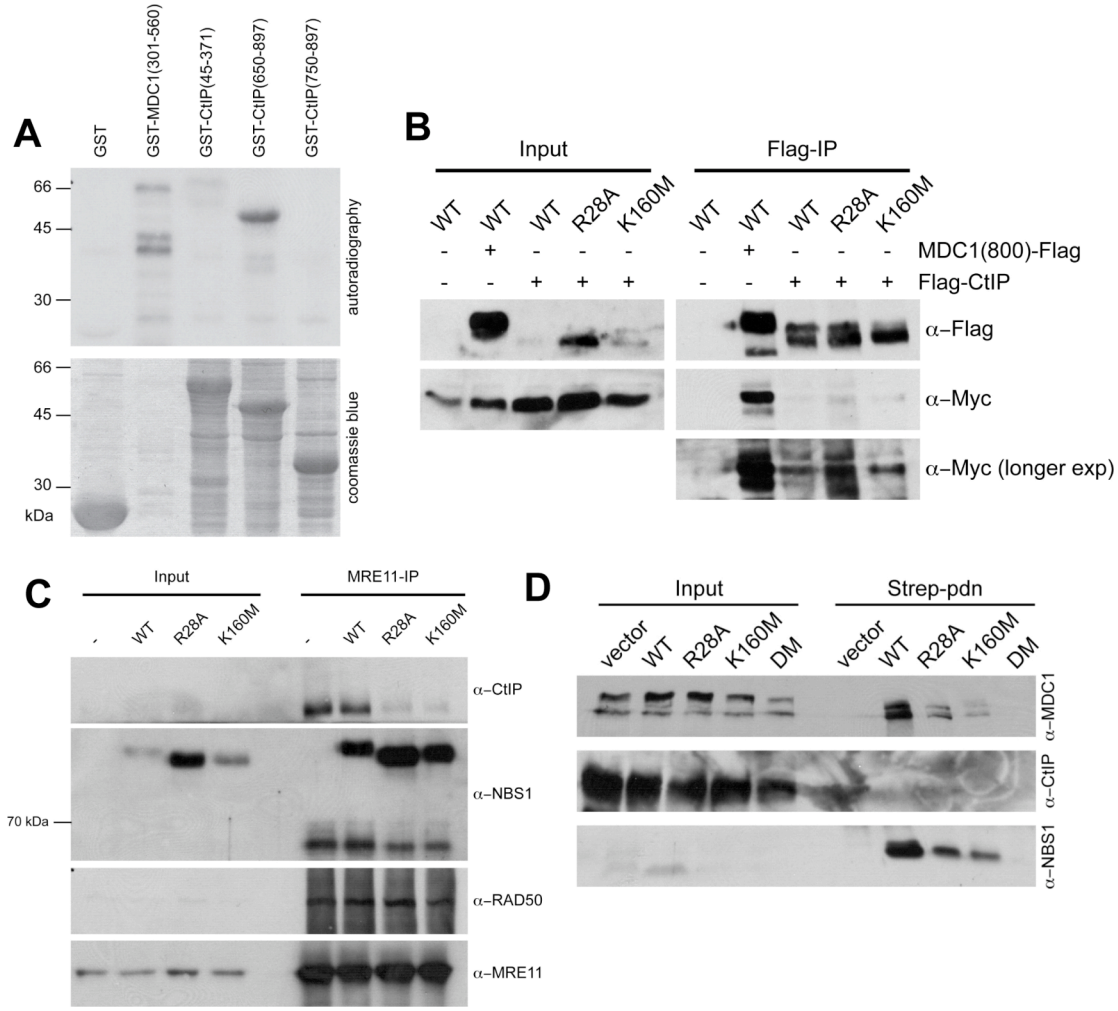

Figure 2.10 The FHA domain of NBS1 does not interact in a phospho-specific manner with CtIP. A) Recombinantly expressed and purified GST-tagged fragments of CtIP were incubated with recombinant CK2 in the presence of γ-32P-ATP, followed by analysis by SDS PAGE and autoradiography. B) HEK 293T cells were transfected with full-length Flag-tagged CtIP (as a control in one reaction with Flag-tagged MDC1) and Myc-tagged NBS1 WT or mutants as indicated. Flag-beads were used for co-immunoprecipitations and Flag and Myc antibodies for detection by Western blot. C) NBS-iLB1 cells reconstituted with either WT or mutant NBS1 were used for an anti-MRE11 immunoprecipitation, followed by SDS PAGE and Western blotting for the indicated proteins. D) HEK 293T cells were transfected with either WT or mutant NBS1(1-382) Strep-tagged fragments. Strep-pulldowns were performed, followed by analysis by SDS PAGE and Western blotting.

To solve the problem that CtIP might also interact with either RAD50 or MRE11 and thus any additional interaction with NBS1 may be “masked”, we Strep-tagged an N-terminal fragment (1-382 aa) of the NBS1 protein containing the FHA and the BRCT domains, but not the MRE11 interaction site. Upon overexpression of this fragment in HEK 293T cells, we

performed Strep-pulldowns and analyzed the fractions for the presence of endogenous CtIP. No traces of CtIP were found in neither of the pulldowns of the NBS1 WT, the FHA domain mutant nor BRCT tandem domain mutant, respectively (Figure 2.10 D). As expected, the positive control MDC1 showed strong interaction with the NBS1 WT fragment but neither with the FHA nor the BRCT domain mutant fragments.

Based on these data, we concluded that CtIP might not directly interact with either the FHA domain or the BRCT tandem domain of NBS1 in a phospho-specific manner, although more comprehensive studies should be performed to proof this interpretation.

2.3 Discussion

The MRN complex is a versatile complex that is involved in various processes of the DDR and was investigated already extensively. Here, we expand the knowledge about the MRN complex by presenting a unique divalent FHA/BRCT-binding mechanism that couples the MRN complex to γH2AX-enriched chromatin regions. Moreover, we show that phospho-binding activities of both the NBS1 FHA domain and BRCT tandem domain are essential for the accumulation of the MRN complex in chromatin adjacent to DSBs *in vivo*. However, only the phospho-binding activity of the FHA domain of NBS1 is required for a functional transient G2/M checkpoint. Furthermore, we did not detect radiosensitivity of either an FHA or BRCT tandem domain mutant. Therefore, we concluded that MDC1-dependent retention of the MRN complex in the γH2AX positive chromatin is not essential for a proper G2/M checkpoint nor is it required for normal survival upon IR. Moreover, we suggest that the NBS1 FHA domain has an additional as yet unidentified binding partner that mediates G2/M checkpoint activation in response to DSBs.

MRN complex recruitment was shown to be dependent on the SDT region of MDC1 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). This acidic repeat region contains six doubly phosphorylated SDT motifs and a minimal set of two of these motifs is necessary for efficient retention of the MRN complex (Melander et al., 2008; Spycher et al., 2008). This suggests that more than one domain of NBS1 may be involved in the association between NBS1 and MDC1. Consistently, we found that both phospho-specific interaction modules present at the NBS1 N-terminus are required for its interaction with MDC1. Since the two phosphorylated residues of the SDT motifs are only separated by one single amino acid, it is unlikely that both domains interact simultaneously with one single motif. This notion is also supported by the recently solved X-ray structure of the globular N-terminal domain containing both the FHA domain and the BRCT tandem domain of NBS1 of *schizosaccharomyces pombe* (Lloyd et al., 2009; Williams et al., 2009). The cleft between the two BRCT domains that binds the phosphate is orientated perpendicular to the pThr-binding loop of the FHA domain. Thus, it is sterically impossible that one NBS1 molecule would bind to one pSDpT motif with both of its phospho-specific domains (Figure 2.11). Despite the low sequence conservation between the yeast and human N-terminal fragment of NBS1 (11 % identity, 28% similarity), it shows structural conservation as investigated by small angle X-ray scattering (Williams et al., 2009). The structural orientation of the FHA domain in respect to the BRCT tandem domain of spNBS1 suggests a distance of 22 aa between two potential binding motifs in the same peptide (Williams et al., 2009). The SDT motifs in MDC1 are between 20 and 40 aa apart from each other. The structure of this region of MDC1 is not known so far, but it is conceivable that its fold brings two motifs in close proximity, although they are farther away in primary structure, to undergo interaction with each of the phospho-specific binding modules of human NBS1.

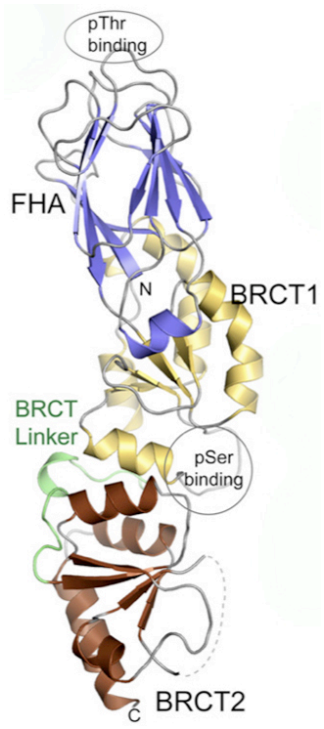

Figure 2.11 The structure of the FHA/BRCT globular domain of NBS1 from s. pombe. The FHA domain is colored in blue, first BRCT domain in yellow, second in brown. Phospho-specific interaction sites are indicated (Williams et al., 2009).

While it is currently unknown why such a divalent binding mechanism has evolved, it is interesting to note that the key phospho-binding residues within the BRCT tandem domain are not conserved in yeast orthologues of NBS1, thus indicating that yeast NBS1 may feature a monovalent phospho-binding module at its N-terminus (Becker et al, 2006). Consistent with this, an N-terminal fragment of human NBS1 but not spNBS1 binds a synthetic pSDpTD-peptide of MDC1 in a stoichiometric ratio greater than 1:1 as measured by isothermal titration calorimetry (ITC) (Lloyd et al., 2009). Furthermore, using a peptide where the phosphorylated threonine was replaced by a phosphorylated serine, the same group could show that only the BRCT tandem domain of human NBS1 is binding to this peptide but neither a BRCT mutant nor wild type spNBS1. Thus, the BRCT tandem domain of spNBS1 seems to lack the phospho-specific interaction properties that normally are displayed by BRCT domains despite its similar folding. Alternatively, the site that is recognised by the BRCT tandem domain of spNBS1 might vary from known BRCT-binding consensus sites (pS/T-X-X-F/Y/D/E) and hence they were not detected so far (Manke et al., 2003; Yu et al., 2003, Williams et al., 2009).

Since there is no clear MDC1 orthologue in yeast, spNBS1 and Xrs2 (NBS1 in fission and budding yeast, respectively), must have other binding partners in these organisms.

It was recently demonstrated that the s. pombe protein Ctp1, the orthologue of the human protein CtIP, directly interacts with spNbs1 in a mechanism that involves phosphorylation of SDT-like motifs in Ctp1 (Lloyd et al., 2009; Williams et al., 2009). Mutation of key residues in the FHA domain of spNBS1 or substitution of phosphorylated threonines to alanine in Ctp1 lead to sensitivity to several DSBs inducing agents. Thus, this interaction seems to be important for DDR in yeast.

This let us to hypothesize that CtIP might be the putative additional binding partner of the human NBS1 FHA domain to mediate its G2/M checkpoint function. First of all, CtIP is involved in the resection of DSBs in S and G2 phases of the cell cycle and was previously shown to be required for the efficient induction of the G2/M DNA damage checkpoint (Yu and Chen, 2004). In addition, CtIP was already shown to interact with the MRN complex, although it is not yet clear how exactly CtIP associates with the MRN complex (Sartori et al., 2007; Chen et al., 2008). A C-terminal fragment containing the last 108 aa of CtIP pull downs the MRN complex as efficiently as the full-length protein (Sartori et al., 2007). Consistently, an N-terminal as well as a C-terminal fragment of CtIP was suggested to interact with all three individual subunits of the MRN complex, which were purified from insect cells in a other study (Yuan and Chen, 2009). In addition, the interaction between CtIP and MRN complex was shown to be independent of DNA damage (Sartori et al., 2007; Chen et al., 2008). Thus, the interaction between NBS1 and CtIP may not only exist in fission yeast, but may be conserved also in higher eukaryotes.

Furthermore, by co-crystallisation of a Ctp1 peptide with the N-terminal FHA/BRCT domain fragment of spNBS1, Williams et al. suggested a unique recognition site for NBS1-FHA that selects for phosphothreonine residues followed by an aspartate at the plus one position, despite the usual selectivity for the +3 amino acid C-terminal to the pThr position (Williams et al., 2009; Durocher et al., 2000). Searching for this minimal interaction site of the NBS1 FHA domain in human CtIP rather than looking for motifs similar to SDT region of MDC1 leads to the identification of one such site in CtIP (Thr430). NetPhos 2.0 predicts a low probability that this site is indeed phosphorylated (with a score of about 0.16 compared to 0.99 for strong predictions). However, Lloyd et al. suggest the selectivity for the spNBS1 FHA domain to be an aspartate or glutamate at pThr(+3) position (Lloyd et al., 2009). CtIP contains several of these T-X-X-D/E motifs, especially in its C-terminus half. Such motifs are consensus sites for phosphorylation by CK2 and indeed, we could show efficient phosphorylation by CK2 of a region containing these sites *in vitro*. However, we did not detect any apparent interaction between NBS1 and CtIP by pulldown experiments of an overexpressed N-terminal fragment of hNBS1 in HEK 293T and neither did we find any unique CtIP peptide after a similar pulldown by highly sensitive mass spectrometry. Either our experiments were not sensitive enough to detect CtIP as a binding factor of the human NBS1 FHA domain, or the selectivity of the FHA domain in human NBS1 might differ from the one in s. pombe. Since we could not detect any interaction between an N-terminal

fragment of hNBS1 and CtIP in cell extracts, we suggest that the FHA domain of human NBS1 does not efficiently interact with CtIP in a phospho-dependent manner. To support this conclusion further, additional experiments need to be performed. For example, artificial phosphopeptides containing these potential interaction sites in CtIP could be synthesized and tested for their ability to interact with human NBS1. For this, both pulldown experiments from cell nuclear extracts and interaction studies with the purified NBS1 FHA/BRCT region (e.g. ITC) may be employed.

As a second attempt to identify an additional binding partner of the FHA domain of NBS1 that mediates the G2/M checkpoint function, we performed tandem affinity purification (TAP) of a N-terminal NBS1 fragment containing both FHA and BRCT tandem domain, followed by mass spectrometry analysis. By following this approach, we found that BRCA1 associates with the N-terminal part of NBS1, as has been published before (Chen et al., 2008).
In synchronised cell extracts, BRCA1 interacts with the MRN complex and CtIP only in G2/M phase of the cell cycle. Complex formation was already detected in untreated cells and the association is only partially stabilised upon DNA damage. The interaction between BRCA1 and the MRN complex depends on CDK activity and on the BRCT domain of BRCA1 that was shown to specifically interact with the phosphorylated residues Ser327 on CtIP (Yu and Chen, 2004). Extracts depleted of CtIP by RNA interference showed impaired NBS1 binding to BRCA1 immunoprecipitates. However, the GST-tagged BRCT domains of BRCA1 could not pull down NBS1. Thus, the interaction of CtIP with the BRCT domains of BRCA1 cannot link the MRN complex with BRCA1. Consistently, the N-terminus of BRCA1 was shown to interact directly with NBS1 (Chen et al., 2008). Hence, all three components are detected in a complex and are probably required for the complex to be stable.
Here we show that BRCA1 interacts with NBS1 in a phospho-specific manner via the FHA domain and BRCT tandem domain of NBS1. The BRCT tandem domain of hNBS1 was proposed to bind specifically to pS/T-X-X-D/E motifs (Williams et al., 2009). BRCA1 contains one TD motif at T231 and several T-X-X-D/E dispersed over the whole primary sequence. However, the interaction of the FHA and the BRCT tandem domain of NBS1 with BRCA1 could also be indirect. Thus, direct interaction has to be shown first by *in vitro* studies. Subsequently, the residues in BRCA1 that are responsible for interaction with the FHA and the BRCT tandem domain could be identified.

NBS1 was shown to be involved in the G2/M checkpoint. Mouse lymphocytes with a conditional knockdown of NBS1 display a strong G2/M checkpoint defect (Difilippantonio et al., 2005). Compared to that, cells derived from NBS patients and cells derived from mice of a humanized mouse model of this disease, respectively show only a partial G2/M checkpoint defect that more pronounced at lower doses of IR. These cells weakly express a truncated version of NBS1: an N-terminal fragment of about 26 kDa and a C-terminal fragment of 70

kDa, encompassing the MRE11 interaction motif and the ATM-binding domain. The expression of the C-terminal part of the protein is sufficient to stabilise the complex and mediate its localisation to the nucleus and hence might also account for the weak G2/M checkpoint defect of these patient cell lines. One research group did not detect any G2/M checkpoint defects at higher doses of 6 Gy in immortalized patient cell lines of NBS (Xu et al., 2001; Xu et al., 2002). Nevertheless, the N-terminal part of the protein seems to be required for the efficient activation of the checkpoint and specially the FHA domain (Difilippantonio et al, 2007). Here, we reproduced this data in reconstituted patient cell lines and showed that only the intact FHA domain but not the BRCT tandem domain is required for an efficient G2/M checkpoint activation.

The radiosensitivity of cells lacking expression of full-length NBS1 was addressed several times in the literatures and all studies concluded that NBS1 is required for cell survival upon IR treatment. More controversial data was obtained when various deletion and point mutations of NBS1 were studied to address the involvement of the FHA domain, BRCT domains or the phosphorylation of NBS1 by ATM. Using simian virus 40-transformed human fibroblast cell line deficient for full-length NBS1 (NBS-iLB1) and stably infected with either a deletion mutant of the FHA domain or the first BRCT domain, Zhao and co-workers found that the FHA domain but not the BRCT domain is dispensable for survival upon IR (Zhao et al., 2002b). Using the same cell lines, Lee et al. found the same defects for the deletion of the BRCT domain. But in contrast to the earlier study, they saw also sensitivity of cells reconstituted with NBS1 harbouring a point mutation in the FHA domain (Lee et al., 2003). This discrepancy is difficult to explain. First of all, one has to keep in mind that deletion mutations can disrupt proper folding of proteins. Since the structure of the N-terminal fragment of yeast NBS1 displays a hydrophobic interface between the two BRCT domains, deletion of just one of these domains might lead to improper folding. Such proteins are usually expressed at very low levels. A deletion mutant of the first BRCT domain displays indeed lower protein levels than wild type NBS1 (Zhao et al., 2002; Lee et al., 2003). Still, this would not explain why a FHA point mutation shows sensitive where a deletion of the FHA domain does not. Horejsi and colleagues published a partial radiosensitivity of a point mutant of the FHA domain in NBS1 transfected NBS-tert cells (hTert-immortalized NBS fibroblasts)(Horejsi et al., 2004).
However, we could not detect radiosensitivity in reconstituted NBS-iLB1 cells with similar expression levels of NBS1 transgene. Since cells defective for checkpoint activation do not necessarily show radiosensitivity, our findings are not in conflict with each other (Xu et al., 2002). Nonetheless, our findings are controversial to the three studies mentioned above. The reason for this might be a cell line specific effect since we used the SV40 transformed NBS-iLB1 patient cell line whereas Horejsi et al. used an hTert immortalized cell line. Secondly, we achieved similar protein levels of the reconstituted NBS1 protein according to Western blot

but also by immunofluorescence (data not shown). If only half of the cells of a population are complemented with the ectopic protein, lower protein levels are detected by Western blotting, and these mixed cell population would probably display a partial survival defect upon IR originated from the untransfected cells as seen in the first two studies. Nevertheless, more experiments have to be performed to elucidate the role of the FHA domain and the BRCT tandem domain in survival and to shed some light on this controversial data.
In yeast, deletion of either the FHA domain alone or together with the BRCT tandem domain of spNBS1 leads to radiosensitivity and sensitivity to other DNA damaging agents such as hydroxyurea, CPT and UV (Williams et al., 2009). Combined point mutations of two key residues of the FHA domain of spNBS1 lead also to sensitivity towards these agents. The FHA domain of yeast NBS1 seems to be more critically required for survival upon DNA damage than the FHA domain of human NBS1.
Taken together, we suggest that the FHA domain of NBS1 is structurally but not functionally conserved between yeast and human.

By using point mutations that abrogate phospho-specific interactions of the FHA and BRCT tandem domain, we presented here for the first time a separation of function of these two domains. Moreover, we could successfully show that the accumulation and retention of the MRN complex in γH2AX-enriched chromatin is not required for G2/M checkpoint activation. Thus, our structure/function analysis of the N-terminus of human NBS1 increased our understanding of some of the mechanisms of the DDR.

3 Sequence-specific double-strand breaks

3.1 Introduction

3.1.1 Spreading of H2AX phosphorylation

The phosphorylation of the histone variant H2AX is generally acknowledged as a marker for DSBs induced by IR since its discovery by Rogakou and colleagues in 1998 (Rogakou et al., 1998). The same group calculated that this mark spreads up to two megabases far into chromatin adjacent to DNA breaks, but exclusively on chromosomal arms that are broken and never to chromosomes, which happen to be in close proximity but are intact (Rogakou et al., 1999).

The regulation and maintenance as well as the functional implication of this γH2AX-positive chromatin domain are still not understood in detail. On the other hand, a huge list of DDR factors has emerged that interact with this domain (see 1.3.1 for more details). These factors are probably involved in a positive feedback loop that helps to assemble and maintain these chromatin domains. Above all, MDC1 a direct binding partner of the γH2AX C-terminal tail positively regulates γH2AX levels. Upon knock down of MDC1 in irradiated U2OS cells, γH2AX levels are significantly decreased, especially at later time points after irradiation (Stewart et al., 2003; Stucki et al., 2005). Moreover, if the BRCT domains of MDC1 that specifically bind to γH2AX are overexpressed, increased γH2AX levels are detected. Thus, the expression level of MDC1 seems to be critical for the establishment and maintenance of the γH2AX chromatin domain.

In general, recruitment of a protein to the γH2AX chromatin domain is addressed by nuclear foci formation upon irradiation using immunofluorescence microscopy. With specialized microscopy equipment recruitment of proteins to the γH2AX chromatin domain can be distinguished from accumulation of proteins in a chromatin microcompartment that contains single-stranded DNA (Bekker-Jensen et al., 2006). Nevertheless, to obtain more insight into the architecture of the chromatin adjacent to DSBs, other ways to look at damaged chromatin organisation have to be developed, since the resolution of foci detection by immunofluorescence and microscopy is restricted to the resolution of light microscopy.

3.1.2 Sequence-specific DSBs

For several years researchers try to shed some light on the chromatin organisation adjacent to DSBs using chromatin immunoprecipitation (ChIP). Hence, sequence-specific DSBs have to be generated to be able to identify precipitated chromosomal DNA by PCR. Several experimental systems to achieve this were published so far. Especially in yeast, the

understanding of chromatin organisation adjacent to DSBs has made tremendous progress in the past years by the fact that yeast contains the endogenous HO-endonuclease cutting only once in the genome. The phenotype of haploid yeast cells is determined by the mating type sequence that they carry at the MAT locus. To switch the mating type, one of the two sequences of the silenced mating type genes, HML or HMR, is transferred to the central MAT locus. This gene conversion begins with a site-specific cleavage at the MAT locus by the endonuclease HO. To study DSB repair at a defined locus, yeast strains with deletions of both silent loci are used to prevent rapid repair via gene conversion. Using a HO-induced DSB system, the γH2AX signal was shown to spread up to 25 kb to each side of the DSB in yeast (Shroff *et al.*, 2004).

Several similar systems were established in mammalian cells, but most of them relied on the introduction of a rare-cutting homing endonuclease site (e.g. I-SceI) into the human genome. In addition, the endonuclease needed to be overexpressed in these cells to achieve induction of site-specific DSBs (Rouet et al., 1994). Unfortunately, these systems were not efficient enough to perform ChIP analysis (< 10% cutting efficiency). Thus, γH2AX spreading was studied in mammalian cells at the telomeres of senescent cells (Meier *et al.*, 2007). However, the question remained whether or not the organisation of the subtelomeric chromatin compartments and their changes upon telomere shortening was the same as the architecture of the γH2AX chromatin domains adjacent to a bona fide DSB. Moreover, systems like I-SceI used so far in mammalian cells usually lead to very few DSBs per genome, which does not allow statistical analyses of γH2AX spreading.

3.1.3 The endonuclease I-PpoI

Since all the systems used so far have their drawbacks, new approaches have to be established to introduce sequence-specific DSBs in the context of chromatin. To this end, Berkovich *et al.* expressed the homing endonuclease I-PpoI in human cells. This enzyme is an intron-encoded endonuclease from slime mould *physarum polycephalum* with a 15 bp long recognition sequence (Muscarella et al., 1990). There exist about 300 I-PpoI recognition sequences in the human genome. Upon expression of I-PpoI in human cell lines, 10 % of the recognition sites are cut and can induce a DDR (Monnat et al., 1999; Berkovich et al., 2007). This number corresponds to the amount of DSBs generated by irradiation with 0.8 Gy, but in contrast to irradiation the endonuclease gives rise to DSBs at specific loci in the genome. By performing ChIP with cells expressing I-PpoI, ATM activation and its recruitment to DSB-flanking chromatin was investigated (Berkovich *et al.*, 2007).

In addition, the expression of the nuclease should be inducible or kept actively out of the nucleus, since its constitutive expression and hence induction of DSBs may lead to cell cycle arrest and/or apoptosis. In the above-mentioned system, this problem is solved by

cytoplasmic sequestration of I-PpoI. Upon induction I-PpoI is transferred to the nucleus where it can induce DSB formation. For that purpose, a modified oestrogen receptor gene was fused to the N-terminus of I-PpoI. The modification of the receptor leads to an transcriptionally inactive receptor upon its binding to the natural ligand oestrogen, but it can bind the artificial ligand 4-hydroxytamoxifen (4-OHT) that leads to its shuttling to the nucleus (Littlewood et al., 1995). Using this naturally occurring induction of nucleus shuttling, the formation of DSBs can be precisely controlled.

We decided to use this system, to elucidate the influence of MDC1 on the regulation of the assembly and maintenance of the γH2AX chromatin domain.

3.2 Results

3.2.1 Characterization of stably transduced U2OS cells expressing HA-ER-I-PpoI

To study the γH2AX spreading and maintenance, an experimental setup was developed to induce sequence-specific DSBs in order to perform ChIP (Figure 3.1). A plasmid harbouring the endonuclease I-PpoI fused to the C-terminus of a modified oestrogen receptor was received from the lab of M. Kastan. U2OS cells transfected with this construct showed weak expression of the HA-tagged endonuclease. Upon addition of the oestrogen receptor ligand 4-OHT for several hours, the protein was stabilised as indicated by higher protein levels on Western blots (Figure 3.2). To address if the fusion protein would induce DSBs as proposed, γH2AX phosphorylation levels were analyzed by Western blotting. Transfection of vector alone already leads to a γH2AX signal, and no additional elevation of the γH2AX levels upon induction by 4-OHT could be detected (Figure 3.2).

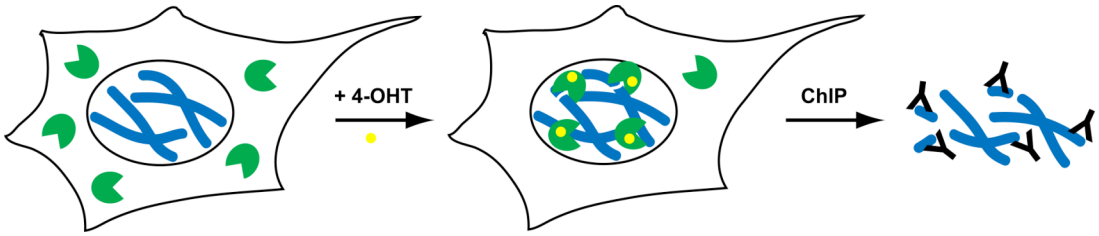

Figure 3.1 The principle of the experimental system. In uninduced cells, the fusion protein HA-ER-I-PpoI (green half moon) is localised to the cytoplasm. Upon binding of 4-OHT (yellow ball) to the oestrogen receptor, the fusion protein shuttles to the nucleus and induces DNA breaks. Chromatinimmunoprecipitation can then be performed to analyse protein accumulation at sites of DSBs.

To circumvent elevated γH2AX levels induced by transient transfection, U2OS cells were transduced with a retrovirus harbouring the fusion construct HA-ER-I-PpoI as it was suggested by Berkovich *et al.* Since the transduction efficiency was low (data not shown), we decided to stably transduce U2OS cells with the fusion construct. Infected cells were cultured in selection medium for up to two weeks, colonies were picked and selected for HA-ER-I-PpoI expression by immunofluorescence. These cell lines consistently express HA-ER-I-PpoI, which localizes to the cytoplasm. Upon addition of 4-OHT the fusion protein is transported to the nucleus (Figure 3.3).

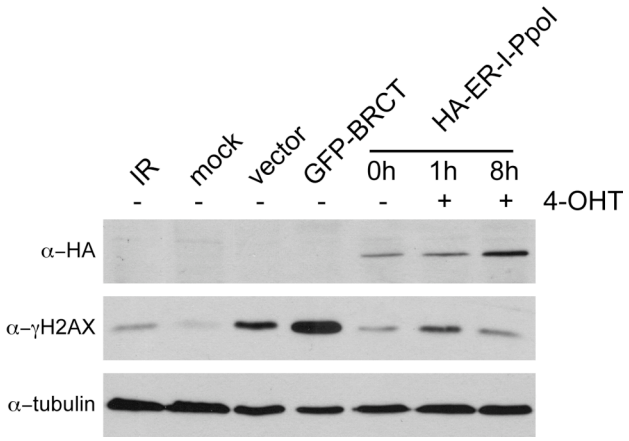

Figure 3.2 Transiently transfection with vector alone leads to elevated γH2AX levels compared to mock transfected cells. U2OS cells were transiently transfected with vector or HA-ER-I-PpoI using MetafectenePro, cultured for 48 hours, induced where indicated by addition of 1 µM 4-OHT, and analyzed by SDS PAGE and Western blotting.

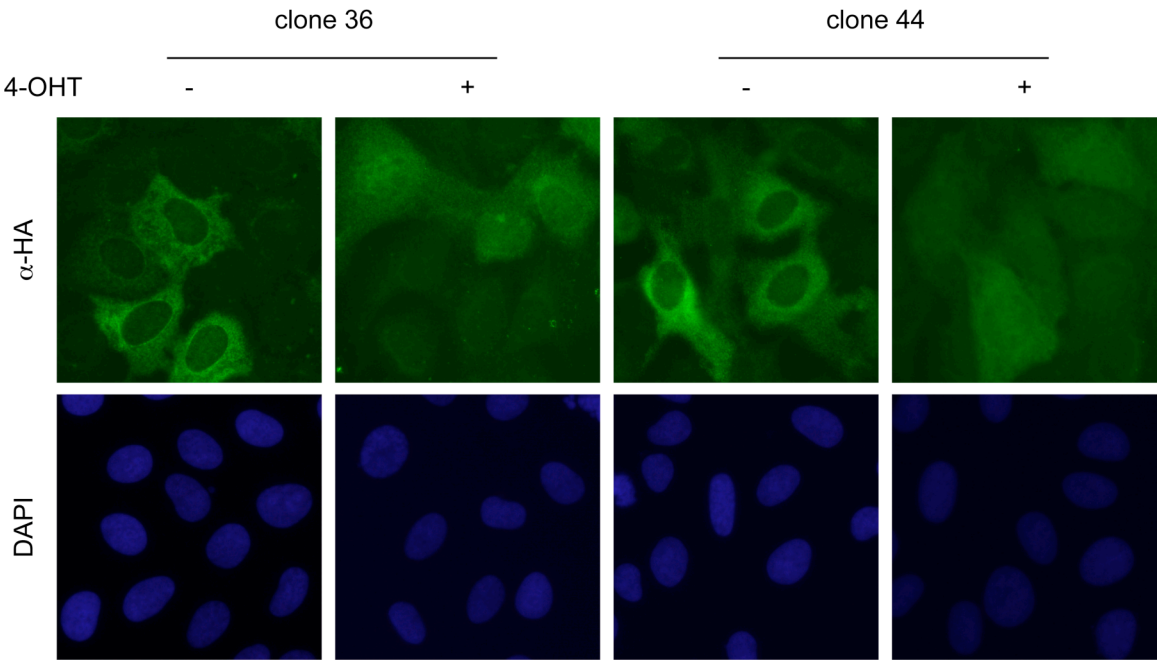

Figure 3.3 The fusion protein HA-ER-I-PpoI shuttles to the nucleus upon 4-OHT addition. Cells were cultured on glass coverslips and incubated overnight with 1 µM 4-OHT where indicated, fixed with ice-cold MeOH and immunostained with an antibody against the HA-tag. Pictures were taken using an epifluoresence microscope.

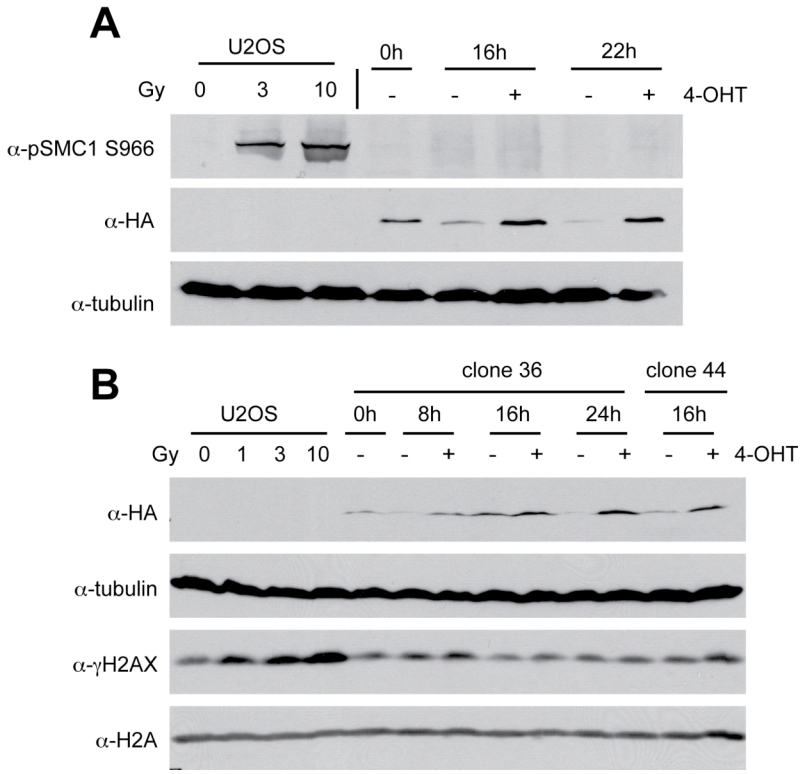

Figure 3.4 Stable I-PpoI-expressing U2OS cells show no phosphorylation of SMC1 or H2AX upon induction. (A) Cells were treated with 1 µM 4-OHT for several hours where indicated. SDS extracts were prepared and protein levels were analyzed by Western blotting. (B) Same as in (A).

Expression levels of HA-ER-I-PpoI in the stably transfected cell lines were also analyzed by Western blotting. In uninduced cells the fusion protein was present at low levels. It may either be expressed only weakly or degraded in the cytoplasm. Induced cells show a stabilisation of the fusion protein between 8 and 16 hours after addition of 4-OHT (Figure 3.4).

Using these stable cell lines, we investigated whether HA-ER-I-PpoI expression and shuttling to the nucleus leads to activation of the DDR by Western blotting. As a read-out phosphorylation of Ser139 of H2AX and phosphorylation of Ser966 of SMC1 were chosen. Both proteins are phosphorylated by ATM in response to DSB induction. Control cells show elevated phosphorylation levels of both proteins upon IR treatment, whereas the stably transfected HA-ER-I-PpoI U2OS cells show no increase in phosphorylation of these proteins upon induction (Figure 3.4).

The same question was also addressed by analysing γH2AX foci formation after induction by immunofluorescence. Since untreated U2OS cells already show weak γH2AX foci, it could not be distinguished between induced and uninduced cells (data not shown). The few DSBs that are produced in these cells seem to be below the detection limit of the assay.

To directly investigate the cutting efficiency of the stably expressed HA-ER-I-PpoI at a specific locus, a Southern blot was performed. An appropriate restriction enzyme was

chosen so that the recognition site of I-PpoI at the locus 1p32.2 of chromosome 1 is on a 6 kb big fragment. When I-PpoI cuts at the site, two 3 kb long fragments are generated; one of them can be detected by the same radioactive probe as the full-length 6 kb fragment. As a control, isolated genomic DNA from U2OS cells were incubated with purified recombinant I-PpoI. In this control reaction, 100 percent cutting efficiency was detected. In contrast in the stably infected I-PpoI U2OS cells no band was detected upon induction by 4-OHT indicating that the stably expressed I-PpoI does not cut at this specific locus (Figure 3.5).

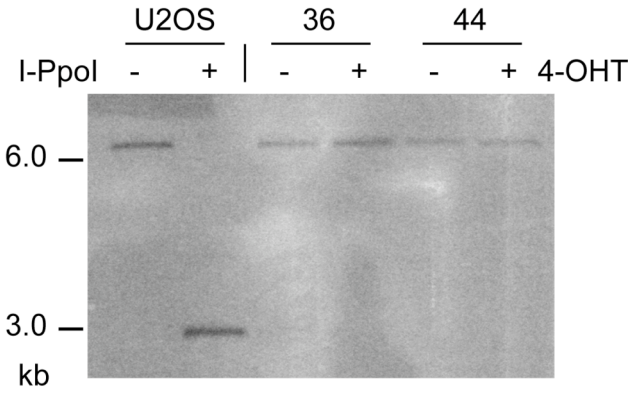

Figure 3.5 Stably expressed I-PpoI in U2OS does not cut at the locus 1p32.2 of chromosome I. Isolated genomic DNA from clone 36 and 44 of the stably expressing I-PpoI cell lines was digested with SacI and analyzed by a Southern Blot using a 32P-labeled probe.

3.3 Discussion

A system that generates sequence-specific DSBs allows studying chromatin organisation at a defined locus by chromatin immunoprecipitation (ChIP). Recently, a system was published where several sequence-specific DSBs could be generated at defined loci in the genome upon expression of the homing endonuclease I-PpoI (Berkovich *et al.*, 2007). To enable induction of the DSBs, the endonuclease was fused to a modified oestrogen receptor to sequester the fusion protein in the cytoplasm. Upon addition of the ligand 4-OHT, it shuttles to the nucleus.

Compared to the study of Berkovich *et al.*, only low infection efficiency was obtained by transient transduction of U2OS cells with a retrovirus harbouring the fusion construct HA-ER-I-PpoI. Therefore, stably transduced cell lines were produced. Stabilisation of the protein levels between 8 – 16 h after induction was obtained as in the study by Berkovich *et al.*, but no activation of the DDR could be detected as indicated by the lack of phosphorylation of H2AX and SMC1 (Fig. 2) or ATM (data not shown) – This is in contradiction to the findings of Berkovich *et al*.

A possible explanation for such differences in the dataset could be that the enzyme expressed in our stably transduced U2OS cell lines is inactive. I-PpoI enzymatic activity was addressed by Southern blotting of one specific I-PpoI site on chromosome 1 1p32.2. No cutting was detected. Expression of a truncated inactive version can be excluded, since the fusion protein runs at the expected size of about 50 kDa on a Western blot. However, we cannot exclude that the ER-I-PpoI fusion protein might be an inactive enzyme. The crystal structure of the endonuclease I-PpoI revealed that it forms a dimer when activated (Flick *et al.* 1997; Flick et al., 1998). The oestrogen receptor fused to the N-terminus of I-PpoI might inhibit this dimerisation and hence I-PpoI activation. To test this hypothesis, the fusion protein could be expressed in a human cell line such as U2OS cells and purified via its HA-tag. Incubation with a plasmid containing the I-PpoI recognition site could reveal cutting efficiency *in vitro*. Alternatively, since restriction is not expected to occur in all the cells at the same time at a specific locus, the percentage of the cell population harbouring a DSB at this site could be too low to be detected by Southern blotting

In addition, DSBs generated by restriction enzymes such as I-PpoI give rise to two sticky, complementary ends. The architecture of such a DSB does not mimic the structure of DSBs induced by irradiation. Therefore, the repair pathways as well as the repair kinetics of such DSBs do probably not reflect what happens in more complicated cases. This is probably the major drawback of studying nuclease-induced DSBs.

A more versatile alternative is to use triplex-forming oligonucleotides coupled with a DSB inducing agent. These oligonucleotides of about 20 bp lengths can be designed against any desired sequence of the human genome. By using locked nucleic acids, the affinity to the

complementary sequences was shown to be very specific and the dissociation constant in the nanomolar range (Brunet et al., 2006). Conjugated with orthophenanthroline these triplex-forming oligonucleotides induce DSBs at defined loci in the human genome and initiate a DDR and even DSB repair (Cannata et al., 2008). Compared to nuclease-induced DSBs, the advantage of such a system is that the induced DSBs resemble more those of IR. Only future studies will reveal if the advantages of such a system will overcome the costly and laborious preparation of the oligonucleotides so that they become a standard technique to study DSB repair.

Recently, two studies were published that give more insight into γH2AX spreading in mammalian cells. By studying the induction of DSBs during development of lymphocytes, Savic and colleagues proposed that the γH2AX signal is dynamic, but maintained over time at a constant level in a defined region rather than it spreading over time into chromatin regions emanating of the break (Savic et al., 2009).
Moreover, these authors found that not only for the establishment but also for the maintenance of the equilibrium of γH2AX density, the activities of the phosphatase PP2A and the ATM kinase are required. Inhibiting ATM led to the same distance of chromatin covered by γH2AX signal but to a lower signal density that was DNA-PK dependent. Together these findings reinforce the hypothesis that the maintenance of the γH2AX chromatin domain is very dynamic and requires repeated cycles of phosphorylation of H2AX by ATM and its dephosphorylation by PP2A.
MDC1's involvement in the establishment and maintenance of this dynamic γH2AX region is not yet resolved completely, although it seems to be required predominantly for the formation of the proximal 100 kb of the γH2AX chromatin domain, where it probably helps ATM to maintain a constant high density of γH2AX (Savic et al., 2009).
Using an endonuclease to induce sequence-specific DSBs followed by ChIP-chip analysis recently yielded a high-resolution map of γH2AX signal distribution throughout the genome (Iacovoni et al., 2010). First, this study showed the anticipated length of 0.5 up to 2 Mb of the γH2AX chromatin domain in mammalian cells. Moreover, it revealed that the distribution of γH2AX is uneven and asymmetric around a given DSB and some boundaries seem to exist that restricted the γH2AX signal to certain chromatin regions. In addition, gene transcription was identified as one of the major determinants that control γH2AX distribution. Pol II binding to chromatin is mutually exclusive with the γH2AX modification. Furthermore, transcription does not seem to be altered by the occurrence of a DSB nearby nor is the γH2AX signal changed in intensity more distal to the transcribed region (Iacovoni et al., 2010).
Using a system such as this, the influence of MDC1 on the γH2AX chromatin domain may be investigated further.

4 Discussion

Assembly and retention of a large number of proteins in damaged chromatin in so-called IRIF is a central feature in the DDR. Analysing IRIF formation is often used as a read-out for the activation of the DDR upon certain stress stimuli and for the involvement of new factors in the DDR. Our knowledge about IRIF architecture and molecular mechanisms involved in their assembly are constantly growing, but it is still not completely understood in which processes of the DDR these protein aggregates are involved in. In this study, we describe a divalent FHA/BRCT-binding mechanism between NBS1 and phosphorylated SDT motifs of MDC1 that leads to the retention of the MRN complex in damaged chromatin. In addition, we show that this retention is not required for the role of NBS1 in the activation of the G2/M DNA damage checkpoint.

The functional implication of the MRN complex in the γH2AX chromatin domain is still not yet clear. MDC1-dependent recruitment of the MRN complex was suggested to be involved in the 'spreading' of the γH2AX epigenetic mark along the chromosome upon DSB formation (Stucki and Jackson, 2006). In this model, ATM is activated at the break site and phosphorylates the most proximal H2AX molecules. Together with MDC1 the MRN complex is recruited to this site. Via its subunit NBS1, the MRN complex recruits more ATM resulting in the subsequent phosphorylation of H2AX molecules that are more distal to the DNA lesions. Due to this positive feedback loop initiated by MDC1, the γH2AX mark could "spread" along the broken chromosome. This model was partially challenged and refined by a study that used ChIP to analyse protein retention in damaged chromatin in lymphocytes derived either from wild type mice or from MDC1 knockout mice (Savic et al., 2009). Interestingly, γH2AX was found in a chromatin region similar in length in MDC1 knockout and in wild type lymphocytes, but the γH2AX density in close proximity to the break was reduced in MDC1-deficient cells compared to wild type cells. Soluble ATM can phosphorylate H2AX along the chromatin axis in an MDC1-independent manner, but for increased concentration of γH2AX adjacent to the break, MDC1 is required. Chromatin retention of ATM through a MDC1/MRN complex-dependent manner could be responsible for the increased concentration of γH2AX adjacent to the break and could explain the requirement of MDC1. However, RAG-induced DSBs in developing lymphocytes only occurs in the immunoglobulin genes. These chromatin regions might differ from other regions and thus, also the distribution of the γH2AX mark and the accumulation of DDR factors might be different.

What is the function of the massive protein accumulation in the γH2AX chromatin domains? Several suggestions can be found in the literature.

MDC1 is the major organizer of the assembly and maintenance of the γH2AX chromatin domain. In the absence of MDC1 other proteins such as NBS1, 53BP1, BRCA1 and RNF8 do not assemble either in IRIF (Lou et al, 2006; Stucki et al., 2005; Huen et al., 2007; Kolas et

al., 2007; Mailand et al., 2007). To get an idea about the function of the accumulation of proteins into IRIF, the phenotypes of MDC1 knock out cells can be examined. Surprisingly, H2AX and MDC1 knock out cells display only relatively mild phenotypes as compared to the loss of other DDR factors. Moreover, both genes are not essential for viability. Cells deficient for H2AX and MDC1 show a partial G2/M checkpoint defect only at low doses (Fernandez-Capetillo et al., 2002; Lou et al., 2006). Furthermore, as we demonstrated here, the recruitment of MDC1 to γH2AX chromatin domains is not required for the activation of the G2/M checkpoint (see Figure 2.7). However, MEFs isolated from MDC1 and H2AX knock out mice show repair defects and hypersensitivity towards IR (Celeste et al., 2002; Lou et al., 2006). These phenotypes implicate the involvement of H2AX and MDC1 in DSB repair.

Studies in yeast and mammalian cells revealed that tethering of repair factors to chromatin in undamaged cells results in recruitment of other repair factors and phosphorylation of these factors (Soutoglou and Misteli, 2008; Bonilla et al., 2008). This suggests that local concentration of certain DDR factors in IRIF facilitates the initiation of the signalling events and the amplification of the signal.

Both 53BP1 and BRCA1 accumulate in foci and are implicated in the appropriate choice of the repair pathways (HR or NHEJ). The two pathways compete with each other for the repair of the lesions. BRCA1-deficient cells display high chromosomal aberrations when treated with camptothecin or PARP inhibitor since the repair of the resulting lesions is highly dependent on HR (Bryant et al., 2005; Farmer et al., 2005). The explanation for the chromosomal instability phenotype is that deficiency in the HR protein BRCA1 leads to illegitimate repair of DNA breaks via NHEJ. Bunting and colleagues recently showed that knock out of 53BP1 in these BRCA1-deficient cells decreases the chromosomal aberration phenotype and the survival of these cells is similar to wild type cells (Bunting et al., 2010). This indicates that 53BP1 inhibits HR in BRCA1-deficient cells and leads to increased repair via NHEJ. Only when 53BP1 is absent, HR can occur in these cells. Thus, 53BP1 and BRCA1 seem to fine-tune the balance between the HR and NHEJ repair pathways.

Evidence arise that the assembly of proteins into foci shelter the broken ends and prevent illegitimate repair. H2AX was shown to preserve the structural integrity of broken DNA by preventing resection of these DNA ends in G1 phase of lymphocytes (Helmink et al., 2010). Since in G1 phase no template is present for HR-mediated repair, resection during this cell cycle phase would lead to significant chromosomal instability. The actual mechanism how H2AX is involved in repressing resection is not yet known. Other proteins present in IRIF might also be involved. MDC1 for instance might sequestrate the MRN complex and retain it at the damaged chromatin region, thereby preventing the MRN complex from interacting with CtIP and BRCA1 to perform resection. A mutually exclusive interaction between the FHA domain of NBS1 with either MDC1 or CtIP would support such a hypothesis. However, we were unable to detect a direct interaction between the FHA domain of NBS1 and CtIP.

Defects in IRIF formation or loss of DDR factors that accumulate in IRIF are frequently associated with genomic instability and cancer. For instance, H2AX knock out mice display chromosomal instability (Bassing et al., 2003; Celeste et al., 2003). Knock out of p53 in these mice results in highly increased cancer incidence. H2AX may therefore be regarded as a tumour suppressor. Furthermore, hypomorphic mutations in many proteins known to accumulate in IRIF lead to predisposition to cancer. Nijmegen breakage syndrome (NBS) for instance results in predisposition to cancer and additionally also in immunodeficiency, microcephaly and growth retardation. Recently, the phenotype of the RIDDLE syndrome was described to be caused by a mutation in the RNF168 gene (Stewart et al., 2007). This E3 ligase is the first protein of the ubiquitin pathway that is described to be mutated in a human disorder (Stewart et al., 2009; Doil et al., 2009). These syndromes underline the importance of the DDR machinery in preventing tumour formation.

Knowledge about the mechanisms involved in the DDR may generate resources for new cancer therapies. The majority of current cancer therapies target the increased cell proliferation of the cancer tissue as compared to the normal tissue. This is achieved by inhibiting the cell cycle or by inducing exogenous DNA damage to interfere with DNA replication. How the cancer cells deal with this DNA damage depends on the integrity of the DNA repair pathways in these cells. One potential strategy of recently developed therapeutic approaches is to sensitize cancer cells to genotoxic drugs by selectively inhibiting DNA repair pathways. In addition, such DNA repair inhibitors can also be implemented to selectively kill tumour cells but not normal cells. Since cancer cells have often inactivated one or the other genome surveillance pathway, these cells are more dependent on certain DDR pathways. Inhibiting these pathways may convert spontaneous DNA lesions into a life-threatening load of DNA damage for the cancer cell. Normal cells may be unaffected since they have another functional DDR pathway that can deal with the DNA damage. In future, increased knowledge about the mechanisms of DNA repair pathways, DSBs signalling and cell cycle checkpoints will hopefully uncover new targets and new protocols for targeted cancer therapy.

5 Appendix

5.1 Material and Methods

Cell lines and cell culture

293T human embryonic kidney cells, simian virus 40-transformed human fibroblast NBS-iLB1 cells and human U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagle's Medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco) and penicillin/streptomycin (100 U/ml; Gibco). NBS-iLB1 cells and NBS-iLB1 cells stably transduced with R28A mutant of NBS1 was a gift from K. Cerosaletti (BRI, Seattle, USA). NBS1-iLB1 stably expressing the wild type, R28A mutant, K160M mutant and R28A/K160M NBS1 as well as U2OS stably expressing I-PpoI were generated by retroviral transduction (RetroMax, Imgenex). The YFP-BRCT-expressing U2OS cell line was described previously (Stucki et al, 2005). Sf9 insect cells were cultured in Grace's insect medium (Gibco) supplemented with 10% fetal calf serum (Gibco). Recombinant MRE11, RAD50 and NBS1 baculoviruses were a kind gift from Vilhelm Bohr (NIA, Baltimore, USA). Bac-To-Bac Baculovirus Expression System (Gibco) was used to generate and amplify recombinant baculoviruses.

Plasmids

The human MDC1 GST-SDT and the YFP-BRCT constructs were described previously (Spycher et al., 2008; Stucki et al., 2005). MDC1(1-800 aa) fragment was cloned into a modified pcDNA3.1-Flag mammalian expression vector (Invitrogen). Myc-NBS1 (Falck et al, 2005) was subcloned into pFastBac transfer vector (Gibco) to generate recombinant NBS1 baculoviruses, into pLPCX to generate retroviral particles, respectively and into the pSG5 vector for *in vitro*-translation assays. Point mutations were introduced using the QuickChange site-directed mutagenesis kit (Strategene). pcDNA-Flag-CtIP was a kind gift of A. Sartori (IMCR, Zurich, Switzerland), pEXPR-IBA103 one of N. Mailand (CPR, Copenhagen, Denmark), and pBABE-HA-ER-I-PpoI one of M. Kastan (St. Jude, Memphis, USA). An oligo containing Flag-HA and subsequently NBS1-NLS were cloned into pEXPR-IBA103 to obtain C-terminal Flag-HA-Strep triple tagging of NBS1.

RNA interference

The siRNA oligonucleotides against endogenous human MDC1 were purchased from Dharmacon (ACAGUUGUCCCCACAGCCCdTdT) and Lipofectamine RNAiMAX (Invitrogen) was used for transfections. All steps were carried out according to the manufacturer's protocol.

Transfections

293T cells were transfected with CaPh transfection method. U2OS cells were transfected with MetafectenePro according to the manufacturer's protocol.

Antibodies

Rabbit polyclonal antibodies used in this study included NBS1 (Novus), SMC1 pS966 (Bethyl), H2A (Upstate), and H3 pS10 (Upstate). Mouse monoclonal included γH2AX (Upstate and Millipore), HA (Covance), tubulin (Sigma), Flag (Sigma), c-myc (Roche), and BRCA1 (Santa Cruz). The CtIP antibody was kind a gift from A. Sartori (IMCR, Zurich, Switzerland). Sheep polyclonal antibodies against MRE11, RAD50 and rabbit polyclonal against MDC1 (889) have been described previously (Goldberg et al., 2003).

Secondary antibodies included peroxidase-linked secondary antibodies (GE Healthcare) for immunoblotting, FITC-, Cy3-, texasred-coupled (Jackson laboratories) and alexa647-coupled (Invitrogen) secondary antibodies for immunofluoresence and alex700-coupled (Invitrogen) secondary antibody for FACS analysis.

Protein purification

HeLa nuclear extract was purchased from Cilbiotech (Mons, Belgium). MDC1 GST-SDT fragment was affinity purified on Glutathione-Sepharose (GE Healthcare Biosciences). Recombinant MRN purification from Sf9 cells was described (Spycher et al, 2008). For *in vitro*-translation of full-length NBS1, the TNT system (Promega) was used. The GST-CtIP fragments were a kind gift from A. Sartori (IMCR, Zurich, Switzerland).

GST-pulldowns

Purified GST-fusion proteins (5 µg) were mixed with 1/5 volume of a standard TNT reaction or 5 µg of purified MRN, respectively. Where indicated, GST fusion proteins were pre-treated with 100 U of CK2 (New England BioLabs). The mixture was incubated at 4°C for 30 min to allow binding. Glutathione sepharose beads, diluted in buffer (1x PBS, 1 mM NaF, 10 mM β-glycerophosphate), were added and the suspension was incubated for further 60 min. The beads were washed with buffer (50mM Tris pH 7.5, 120 mM NaCl, 1 mM DTT, 0.2% NP-40) and resuspended in SDS loading buffer. Samples were analyzed with SDS PAGE and Western blotting.

Strep-pulldowns

HEK 293T cells were transiently transfected with NBS1(1-382)-strep WT or mutants, harvested with trypsin and lysed in NTEN buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 1 mM PMSF, 1 mM NaF, 10 mM β-glycerophosphate). 2 mg of lysate were mixed with 40 µl equilibrated Streptavidin-dynabeads (Invitrogen). After 4 hours

incubation on rotator at 4 °C, beads were washed with NTEN buffer and resuspended in SDS loading buffer. Samples were analyzed by SDS PAGE and Western blotting.

Immunoprecipitation

HEK 293T cells were co-transfected with a Flag-tagged fragment of MDC1 (1-800 aa) or full length Flag-CtIP, respectively and Myc-tagged NBS1 constructs. Cells were lysed in lysis buffer (25 mM Tris pH 7.5, 40 mM NaCl, 2 mM $MgCl_2$, 0.5 % NP-40, protease and phosphatase inhibitors, 25 U/ml benzonase (Novagen)) and incubated for 30 min at 4 °C. The concentration of NaCl was increased to 450 mM, and the extracts were incubated for another 30 min at 4 °C. After centrifugation, extracts were diluted to 100 mM NaCl, added to pre-blocked anti-Flag(M2)-beads (Sigma) and incubated for 3 h at 4 °C. The beads were washed with IP-buffer (25 mM Tris pH 7.5, 100 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM DTT, 0.5 % NP-40, protease and phosphatase inhibitors) and resuspended in SDS loading buffer. All samples were analyzed by SDS PAGE and immunoblotting. Immunoprecipitations of reconsituted NBS-iLB1 cell lines were performed as described above, with the exception of increased salt concentration in the IP-buffer (150 mM NaCl). Anti-MRE11 antibody was mixed first with the IP-extracts for 2 h, before addition of pre-blocked G-protein coupled sepharose beads and further incubation of 2 h. To investigate the interaction between the FHA domain of NBS1 and BRCA1, HEK 293T cells were transfected with NBS1(1-382)-Flag-HA-Strep and anti-Flag immunoprecipitations were performed as described above.

Kinase assay

GST-MDC1 fragment or GST-CtIP fragments, respectively coupled to Glutathione beads, were washed with washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 0.2 % NP-40, phosphatase and protease inhibitors) and then incubated for 30 min at 30 °C in kinase buffer (40 mM Tris pH 7.5, 8 mM $MgCl_2$) with CK2 (New England BioLabs) in the presence of γ-^{32}P-ATP. Reactions were stopped by adding SDS PAGE loading buffer and analyzed by SDS PAGE and autoradiography.

Generation of DNA damage

Ionizing radiation treatment was performed in a Faxitron X-ray cabinet system Model 43 with open lid of the cell culture dish.

DSBs in defined nuclear volumes were induced by laser microirradiation with a MMI CELLCUT system containing a 355 nm UVA laser (55 Hz, Molecular Machines & Industries, Switzerland) of cells that were pretreated with 10 µM BrdU for 24 h. The MMICELLTOOLS software with MMIUVCUT plug-in assisted the laser operation using an energy output of 50%. Cells were recovered for 30 – 60 min, fixed with either MeOH or 4 % PFA (YFP-

positive cells) and subjected to immunofluoresence. Images were captured on a Leica SP2 confocal microscope (Leica Microsystems) with a 40x (oil immersion, NA 1.25) objective.

Immunofluorescence

Cells were grown on glass coverslips and treated as anticipitated. Cells were then fixed with MeOH, blocked for at least 30 min with 10% FCS/PBS, stained with antibodies against protein of interest for at least 1 hour up to overnight, and incubated with secondary antibody for 30 min. GFP-positive cells were fixed in 4 % PFA/PBS solution instead, permeabilized in 0.25 % TritonX100/PBS and treated as mentioned above. Coverslips were mounted on glass slides with vecta shield containing DAPI, fixed with nail polisher and analyzed using a epifluorescense microscope (OLYMPUS) or a confocal microscope (Leica Microsystems).

G2/M checkpoint assay

G2/M checkpoint analysis of NBS fibroblasts was done as described (Xu et al, 2002). Briefly, cells were irradiated with a Faxitron X-ray cabinet at the indicated doses during the exponential growth phase. 1h later, cells were harvested, fixed with 70% ethanol/PBS and incubated over night at -20°C. After permeabilization with 0.25% TritonX100/PBS, cells were stained with anti-phospho-histone H3 (Upstate), followed by secondary FITC-coupled anti-rabbit (Jackson) or Alexa 700 (Invitrogen) antibodies and propidium iodide. Data were acquired with a Becton Dickinson flow cytometer or a Beckham Coulter CyAn ADP 9 Color flow cytometer.

Colony formation assay for survival

400 cells of reconstituted NBS-iLB1 cells were seeded to a 6-well plate in triplicates. The next day, cells were irradiated with 3, 5 and 8 Gy, respectively or left untreated and incubated for another 8 days at 37 °C, 5 % CO_2. Cells were then washed once with PBS, fixed for 10 min in ice-cold MeOH and stained for 10 min with crystal violet solution (0.5 % crystal violet in 20 % EtOH). After washing and drying, colonies were counted by eyes. Survival fractions were calculated as the mean cloning efficiency after treatment corrected for plating efficiency.

Isolation of genomic DNA from U2OS cells

Cells were cultured on 15 cm plates and incubated with 1 µM 4-OHT where indicated for the indicated time. Cells were washed with PBS, trypsinized and collected at 1500 rpm for 2 min. The cell pellet was washed with PBS and stored at – 80 °C. For DNA isolation, the thawed cell pellets were incubated for about 5 hours at 55 °C in 700 µl of lysis buffer (50 mM Tris, pH 8.0; 100 mM EDTA; 100 mM NaCl; 1 % SDS) including 350 µg proteinase K, and then mixed for 5 min on the eppendorf mixer. After addition of 250 µl of 5 M NaCl, the

reaction was mixed again and then centrifuged for 10 min at 14 krpm at 4 °C. The supernatant was transferred to a new eppendorf tube and the DNA was precipitated by addition of 500 µl of isopropanol and 2 min mixing on the eppendorf mixer. DNA was pelleted by centrifugation, washed with EtOH, dried for 1 hour at 37 °C and resuspended in ddH_2O for 1 hour at 37 °C.

Southern blot probe cloning and labelling

A region of 683 bp on the 5' site of the I-PpoI site on chromosome I (al391826) was amplified by PCR using the two primers: CGGAATTCGACCACATACATGCGGCCATGG and CGGAATTCTAGCTTCATAAAGAGGCTCTAGGG. PCR product was digested with EcoRI and cloned into the vector pBluescript-KSII+. Plasmid was amplified with a Midiprep using Nucleobond PC-100 (Macherey-Nagel) and digested with EcoRI. Southern blot probe was gel purified using Nucleospin (Macherery-Nagel). Probe was then labelled with the nick-translation kit (Invitrogen) according manufacturer's protocol with the exception that 162.5 pmol of ^{32}P-α-dATP was used with the specific activity of 400 Ci/mmol.

Southern blot

Isolated genomic DNA of the stably transfected U2OS cells with HA-ER-I-PpoI was digested with the restriction enzyme SacI overnight at 37 °C (20 µg DNA and 80 U of enzyme). As a control genomic DNA from U2OS cells was pretreated with recombinant I-PpoI (Promega) for 4h at 37 °C and then subjected to digestion with SacI. DNA was mixed with 10x loading buffer, incubated for 2 – 3 min at 56 °C and loaded onto a 0.7 % TAE agarose gel. The gel was run at 95 V for about 8 hours. Depurination was performed in 0.25 M HCl for 10 min, followed by denaturation in 0.5 M NaOH/1.5 M NaCl for 30 min. A blotting tower was built as follows: a glass plate was laid on a tray filled with denaturation buffer, a Whatman filter paper contacting buffer on both sides was put onto the glass plate, 2 Whatman papers in the size of the gel, the DNA agarose gel upside down, the nylon membrane (Hybond XL, Amersham), 2 Whatman papers in the size of the gel and a stock of paper towels of about 5 cm in height were added on top. A metal block was added as weight to compress the blotting tower. After blotting overnight, the membrane was soaked twice in 2x SSC and then incubated at 80 °C for 1 h to crosslink DNA to the membrane.

Pre-hybridization of the membrane was performed at 65 °C for 1 h on a rotating wheel in 10 ml hybridization buffer (0.25 M Na_2HPO_4, pH 7.2; 7 % SDS) containing 100 µg/ml salmon sperm DNA. 10 – 40 Mcpm of labelled probe was added in hybridization buffer and incubated overnight at 65 °C. The membrane was then rinsed first and then washed twice with washing solution I (40 mM Na_2HPO_4, pH 7.2; 5 % SDS) followed by three times washing with solution II (40 mM Na_2HPO_4, pH 7.2; 1 % SDS). All washing steps were performed at 65 °C in a glass beaker. Radioactive bands were detected by phosphoimaging.

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5.3 Paper

A divalent FHA/BRCT-binding mechanism couples the MRE11-RAD50-NBS1 complex to damaged chromatin

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A divalent FHA/BRCT-binding mechanism couples the MRE11–RAD50–NBS1 complex to damaged chromatin

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The MRE11–RAD50–NBS1 (MRN) complex accumulates at sites of DNA double-strand breaks in large chromatin domains flanking the lesion site. The mechanism of MRN accumulation involves direct binding of the Nijmegen breakage syndrome 1 (NBS1) subunit to phosphorylated mediator of the DNA damage checkpoint 1 (MDC1), a large nuclear adaptor protein that interacts directly with phosphorylated H2AX. NBS1 contains an FHA domain and two BRCT domains at its amino terminus. Here, we show that both of these domains participate in the interaction with phosphorylated MDC1. Point mutations in key amino acid residues of either the FHA or the BRCT domains compromise the interaction with MDC1 and lead to defects in MRN accumulation at sites of DNA damage. Surprisingly, only mutation in the FHA domain, but not in the BRCT domains, yields a G2/M checkpoint defect, indicating that MDC1-dependent chromatin accumulation of the MRN complex at sites of DNA breaks is not required for G2/M checkpoint activation.

Keywords: DNA double-strand breaks; chromatin; NBS1; MDC1; G2/M checkpoint

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INTRODUCTION

Nijmegen breakage syndrome (NBS) is a rare autosomal genetic disorder. NBS patients suffer from growth retardation, microcephaly, dismorphal features, immunodeficiency and predisposition to cancer, mainly lymphomas. Cells derived from NBS patients are radiosensitive, show chromosomal instability and cell-cycle checkpoint, as well as apoptotic defects (van der Burgt *et al*, 1996).

The NBS gene codes for a 754-amino-acid protein named NBS1 (p95; nibrin). It exists exclusively in a complex with two enzymes: MRE11, a structure-specific nuclease, and RAD50, an ATPase/adenylate kinase. Together, these three proteins form the MRE11–RAD50–NBS1 (MRN) complex, a conserved and essential DNA-damage response (DDR) factor that functions in many cellular processes involving DNA double-strand breaks (DSBs), including DSB repair, checkpoint signalling, DNA replication, meiotic recombination and induction of apoptosis (Stracker *et al*, 2004; Difilippantonio & Nussenzweig, 2007).

The MRN complex accumulates at sites of DSBs in large microscopically discernible subnuclear structures, usually referred to as DNA-damage foci. The functional implication of this massive accumulation at sites of DSBs is not yet fully understood. We and others showed recently that focus formation by the MRN complex is mediated by a direct interaction between NBS1 and phosphorylated mediator of the DNA damage checkpoint 1 (MDC1), which is a large nuclear adaptor protein that specifically recognises phosphorylated H2AX (γH2AX; Chapman & Jackson, 2008; Melander *et al*, 2008; Spycher *et al*, 2008; Wu *et al*, 2008). The interaction between NBS1 and MDC1 is dependent on the amino-terminal portion of NBS1 that contains the FHA domain and interacts directly with a constitutively phosphorylated acidic repeat region in MDC1, the SDT repeat (Chapman & Jackson, 2008; Melander *et al*, 2008; Spycher *et al*, 2008). The SDT repeat region is characterized by conserved patches of 8–10 amino acids comprising serine and threonine residues typically separated by an aspartate and embedded further in an acidic sequence environment. This SDT region (referred to as the SDTD region in some papers) interacts with the MRN complex in a phosphorylation-dependent manner. In human MDC1, six SDT motifs were identified and deletion of at least five of them leads to complete abrogation of MRN foci formation (Melander *et al*, 2008; Spycher *et al*, 2008). Analysis of NBS1 recruitment to sites of DSBs showed that on expression of an MDC1 version lacking the SDT regions, NBS1 only accumulates in micro-foci and is not found in the broader chromatin compartments usually covered by γH2AX and MDC1 (Chapman & Jackson, 2008). This indicates that the MRN complex is recruited to DSBs in an MDC1-independent manner, but its sustained interaction with the DSB-flanking chromatin requires MDC1.

Interestingly, MDC1 and MRN exist in a complex even in undamaged cells. This interaction is dependent on the activity of the acidophilic casein kinase 2 (CK2), for which the SDT motifs form consensus phosphorylation sites (Spycher *et al*, 2008; Wu *et al*, 2008). Both serine and threonine residues in each SDT motif are phosphorylated by CK2 *in vivo* and only doubly phosphorylated pSDpT motifs are able to mediate the interaction with NBS1 (Melander *et al*, 2008; Spycher *et al*, 2008).

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No structural information of full-length NSB1 is yet available, but recent nuclear magnetic resonance (NMR) structural data suggested that besides the FHA domain, NBS1 might also feature a tandem BRCT domain at its N terminus (Xu *et al*, 2008). Similarly to FHA domains, tandem BRCT domains have been shown to act as phospho-specific protein–protein interaction modules (Glover *et al*, 2004).

Here, we present evidence that both the NBS1 FHA domain and the tandem BRCT domain interact specifically with phosphorylated MDC1. We show that single point mutations in key residues in both the FHA and the tandem BRCT domain of NBS1 disrupt the interaction with MDC1 and abrogate the accumulation and retention of the MRN complex at sites of DSBs. Surprisingly, only a mutation in the FHA domain induces a significant G2/M DNA-damage checkpoint defect, whereas mutation in the tandem BRCT domain does not. Thus, our findings indicate that MDC1-mediated accumulation of the MRN complex at sites of DSBs is not required for G2/M checkpoint activation and strongly suggest that the FHA domain of NBS1 might have additional, as yet unidentified, interaction partners that mediate G2/M checkpoint activation in response to DSBs.

RESULTS AND DISCUSSION

Both FHA and BRCT domains of NBS1 interact with MDC1

Until recently, sequence comparison and structure predication algorithms indicated that the N-terminal region of NBS1 contained an FHA domain and one single BRCT domain (reviewed in D'Amours & Jackson, 2002). Three years ago, a second putative BRCT domain at the N terminus of NBS1 was discovered by means of a refined bioinformatic analysis (Becker *et al*, 2006). The existence of two BRCT domains downstream from the FHA domain at the NBS1 N terminus was partly confirmed by a recently published NMR structure of the second BRCT domain (Xu *et al*, 2008). Interestingly, there seems to be no spacer between the FHA domain and the putative tandem BRCT domain, indicating that these domains might form one single compact globular structure (Fig 1A). Moreover, conservation of key phospho-binding amino-acid residues in the BRCT tandem domain suggests that like the FHA domain, it might act as a phospho-specific protein–protein interaction module.

We and others have shown recently that the FHA domain of NBS1 associates directly with a constitutively phosphorylated region in MDC1, the SDT repeat region (Chapman & Jackson, 2008; Melander *et al*, 2008; Spycher *et al*, 2008). Mammalian MDC1 contains a total of six SDT motifs, and at least three of these are required for efficient MRN accumulation at sites of DSBs (Spycher *et al*, 2008). This might indicate that more than one binding site with affinity to the phosphorylated SDT region might exist in NBS1. Thus, we tested whether the intact NBS1 BRCT tandem domain was required for efficient association of NBS1 with the full-length phosphorylated SDT region. We phosphorylated (or mock-treated) the human glutathione-*S*-transferase (GST)-tagged MDC1 SDT fragment and assessed its ability to interact with *in vitro*-translated full-length NBS1 protein that carried point mutations in key residues in its phospho-binding FHA and BRCT tandem domains, respectively. As shown before, full-length wild-type NBS1 interacted efficiently with the phosphorylated SDT region of MDC1 (Fig 1B; Melander *et al*, 2008; Spycher *et al*, 2008). Interestingly, FHA domain single mutant (R28A) and a BRCT tandem domain single mutant (K160M) also showed residual SDT-binding activity. However, a double phosphopeptide-binding mutant (R28A/K160M) failed to bind to the phosphorylated SDT region (Fig 1B). This indicates that both the FHA domain and the BRCT tandem domain are able to interact with the phosphorylated MDC1 SDT region *in vitro*.

NBS1 does not exist on its own in the nuclei of mammalian cells, as it is always associated with MRE11 and RAD50. Thus, our assay conditions with the *in vitro*-translated NBS1 do not accurately reflect a physiological situation where NBS1 is part of a heterotrimeric complex. Therefore, we co-expressed all three subunits of the MRN complex in insect cells and tested their binding affinity to the phosphorylated SDT region of MDC1. Also in the context of the intact MRN complex, wild-type NBS1 bound efficiently to the phosphorylated SDT region (Fig 1C). Surprisingly, neither the FHA mutant (R28A) nor the BRCT tandem domain mutant (K160M) was able to associate with the phosphorylated SDT region (Fig 1C). This indicates that when NBS1 exists in a heterotrimeric complex with MRE11 and RAD50, both the intact FHA domain and the BRCT tandem domain of NBS1 are essential for efficient association with phosphorylated MDC1. It is not clear why the NBS1 single mutants interacted with the phosphorylated SDT region when translated *in vitro* but did not in the context of the heterotrimeric MRN complex. However, it is possible that when NBS1 is an integral part of the MRN complex, its N-terminal phosphopeptide-binding region might be sterically less accessible so that efficient association with the SDT region is only possible when both the FHA domain and BRCT tandem domain are contributing to the interaction.

As an intact NBS1 FHA domain and a BRCT tandem domain seem to be essential for interaction with the MDC1 SDT region, we next asked if both of these domains were also involved in complex formation with MDC1 in mammalian cell extracts. We co-expressed a Flag-tagged 800-amino-acid N-terminal fragment of MDC1 (containing the SDT region) with Myc-tagged full-length NBS1 wild type and a mutant derivative, respectively, and tested their association by co-immunoprecipitation. Significantly, only wild-type NBS1 interacted with the MDC1 fragment in extracts prepared from the transfected cells, whereas neither the FHA and the BRCT tandem domain single-mutants (R28A; K160M) nor the double mutant (R28A/K160M) showed any significant binding activity towards MDC1 (Fig 1D).

The BRCT domains of NBS1 are required for MRN foci

Next, we investigated whether the K160M mutation in the BRCT tandem domain would also compromise the accumulation of the MRN complex at sites of DSBs, as observed earlier for the FHA domain mutant R28A (Cerosaletti & Concannon, 2003; Lukas *et al*, 2004). We generated NBS-iLB1 fibroblast cell lines stably transduced with wild-type and mutant NBS1 (supplementary Fig S1A online). Then, we assessed nuclear foci formation of NBS1 in these cell lines by immunofluorescence microscopy. In NBS-iLB1 parental fibroblasts, no NBS1 staining was observed (Fig 2A, top row). However, 81% of the cells stably transduced with wild-type NBS1 showed focal accumulation of NBS1 1 h after irradiation at 5 Gy. By contrast, only 20% of cells stably transduced with R28A NBS1 and 13% of cells stably transduced with K160M NBS1, had a focal NBS1 staining pattern (Fig 2A), thus indicating that sustained interaction of

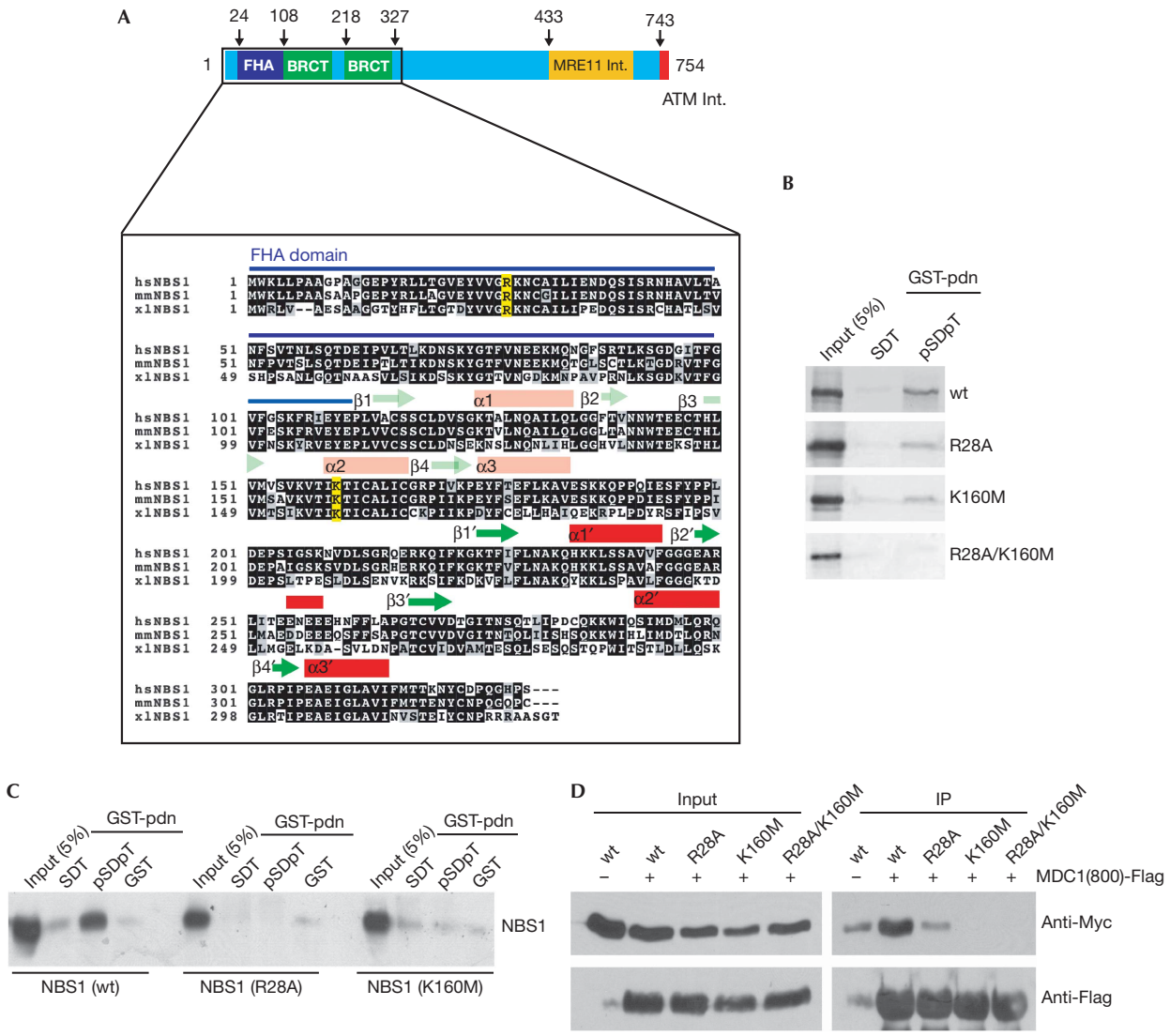

Fig 1 | Both the FHA domain and the tandem BRCT domain of NBS1 are required for the interaction with the phosphorylated SDT region of MDC1 *in vitro*. (**A**) Schematic representation of full-length human NBS1 and its domain composition. The enlarged area shows a sequence alignment of the FHA and BRCT domains of human, mouse and *Xenopus* NBS1. The putative secondary structure of the first (amino-terminal) BRCT domain is indicated by pale colours. The secondary structure of the second (carboxy-terminal) BRCT domain (indicated by bright colours) was derived from Xu *et al* (2008). Phospho-interacting amino acids are highlighted in yellow. (**B**) Purified MDC1 GST-SDT fragment was preincubated with CK2 and ATP. The fragment was then incubated with *in vitro*-translated ^{35}S-labelled NBS1 wild type or mutants for 1 h, washed and resolved by SDS–PAGE and autoradiography. (**C**) Purified MDC1 GST-SDT fragment was preincubated with CK2 and ATP. The fragment was then incubated with purified MRN complex where the NBS1 subunit was either wild type or contained a point mutation in the FHA domain (R28A) or in the BRCT tandem domain (K160M). Bound proteins were separated by SDS–PAGE followed by immunoblotting. The blots were probed with a polyclonal antibody against NBS1. (**D**) Human embryonic kidney 293T cells were transiently transfected with Flag-tagged MDC1(800) fragment and Myc-tagged NBS1 wild type and mutants, as indicated. Flag antibodies were used for co-immunoprecipitation and Myc antibodies for western blot analysis. CK2, casein kinase 2; GST, glutathione-*S*-transferase; IP, immunoprecipitation; MDC1, mediator of the DNA damage checkpoint 1; MRN, MRE11–RAD50–NBS1 complex; NBS1, Nijmegen breakage syndrome 1; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; wt, wild-type.

MRN complex with damaged chromatin requires the phosphopeptide-binding capacity of both the FHA and tandem BRCT domains of NBS1.

To develop these findings further, we used UV-laser microirradiation to induce DSBs in subnuclear volumes (Lukas *et al*, 2004). Under these conditions, wild-type NBS1 accumulated throughout the micro-irradiated nuclear compartments (Fig 2B). However, both the R28A and K160M mutation prevented binding of NBS1 to the γH2AX-coated areas, except for a small fraction of the protein scattered along the irradiated path

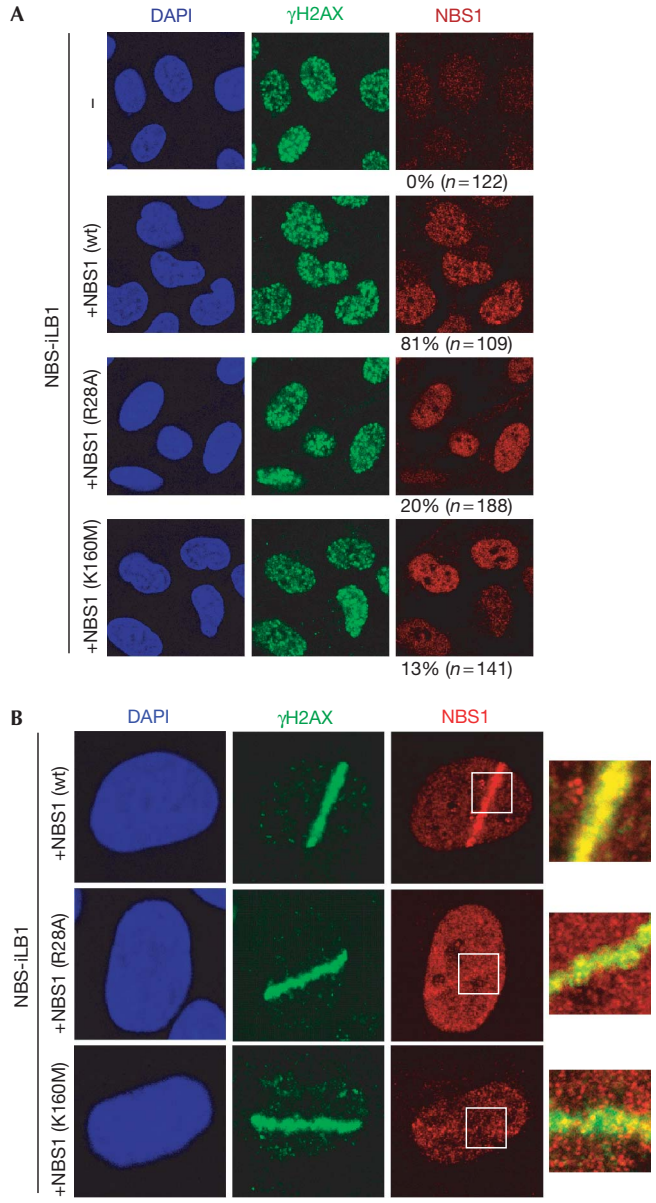

Fig 2 | The BRCT tandem domain of NBS1 is required for focal accumulation of the MRN complex at sites of DSBs *in vivo*. (**A**) NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild-type, R28A or K160M mutant NBS1, respectively, were irradiated at 5 Gy. The irradiated cells were incubated for 1 h, fixed with methanol and probed with the indicated antibodies. Cells were then analysed by confocal microscopy and nuclear foci-positive cells were counted for statistical evaluation. (**B**) NBS-iLB1 fibroblasts stably transduced with wild-type, R28A or K160M mutant NBS1, respectively, were micro-irradiated as described in Methods. The irradiated cells were incubated for 1 h, fixed with methanol and probed with the indicated antibodies. Cells were then analysed by confocal microscopy. DAPI, 4′,6-diamidino-2-phenylindole; MRN, MRE11–RAD50–NBS1 complex; NBS1, Nijmegen breakage syndrome 1.

(Fig 2B, enlarged areas). This indicates that phospho-specific binding of both the NBS1 FHA domain and the BRCT tandem domain to the MDC1 SDT region is essential for efficient accumulation and retention of the MRN complex in damaged nuclear areas.

G2/M checkpoint does not require BRCT domains of NBS1

We proposed previously that MDC1-mediated accumulation of the MRN complex in chromatin regions flanking DSBs was required for efficient activation of the G2/M DNA-damage checkpoint. This was on the basis of the observation that point mutations in the FHA domain that disrupt its phospho-specific binding show partial G2/M checkpoint defects both in human and mouse cells (Difilippantonio *et al*, 2005, 2007; Spycher *et al*, 2008). If this interpretation was correct, we would predict that the K160M mutation in the NBS1 BRCT tandem domain also leads to a G2/M checkpoint defect similar to the R28A FHA mutation, because MDC1-binding and chromatin accumulation are as severely compromised in the K160M mutant as they are in the R28A mutant (see above). Surprisingly, however, we found that several independent clones of NBS fibroblasts stably transduced with K160M NBS1 activated the G2/M checkpoint almost as efficiently as wild-type NBS1 (Fig 3; supplementary Fig S1B online). This indicates that MDC1-binding and MDC1-mediated accumulation of the MRN complex at sites of DSBs are not required for activation of the G2/M checkpoint.

MRN foci formation is not required for the G2/M checkpoint

To verify the aforementioned conclusion, we exploited an earlier observation that overexpression of a C-terminal fragment of MDC1 comprising its γH2AX-binding C-terminal BRCT domains yielded a strong dominant-negative effect on the accumulation and retention of the DDR proteins at sites of DSBs (Stucki *et al*, 2005). We reasoned that if our conclusion was correct, we should not observe a G2/M checkpoint defect on overexpression of the MDC1 BRCT domains. To test this, we used a U2OS cell line carrying a stably integrated, tetracycline-regulated, expression cassette directing the expression of the MDC1 tandem BRCT domain fused to yellow fluorescent protein (YFP). As observed previously (Stucki *et al*, 2005), induction of YFP-BRCT expression by the tetracycline analogue doxocycline (DOX) completely abrogated MRN accumulation at sites of DSBs, as reflected by both NBS1 foci formation (Fig 4A) and UV-laser micro-irradiation (Fig 4B). However, induction of YFP-BRCT expression did not trigger a measurable G2/M checkpoint defect after 1 and 3 Gy of irradiation, respectively (Fig 4C). Significantly, however, down-regulation of endogenous MDC1 in this cell line still yielded a significant G2/M checkpoint defect, irrespective of whether YFP-BRCT expression was induced or not, thus supporting the previous observation that MDC1 is required for G2/M checkpoint activation (Lou *et al*, 2003, 2006; Stewart *et al*, 2003). These data thus support our conclusion that MDC1-mediated accumulation and retention of the MRN complex at sites of DSBs is not required for activation or maintenance of the G2/M checkpoint response.

Speculation

Here, we present a unique divalent FHA/BRCT-binding mechanism that couples the MRN complex to γH2AX-enriched chromatin regions that mark sites of DSBs, and we show for the first time to our knowledge that phospho-binding activities of both the NBS1 FHA domain and the BRCT tandem domain are essential for focal accumulation of the MRN complex at sites of DSBs *in vivo*. It is

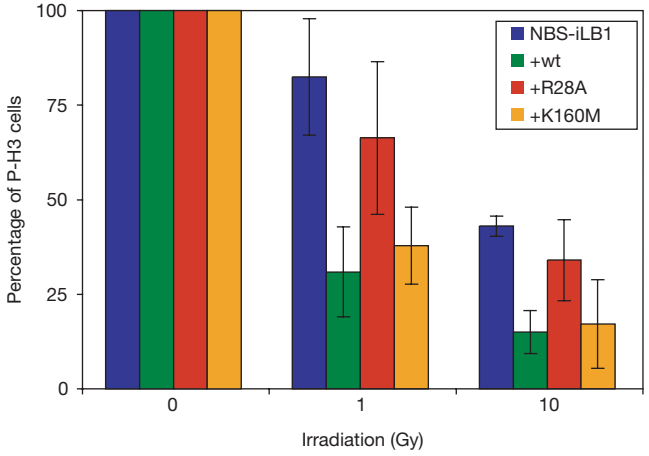

Fig 3 | Mutation in the tandem BRCT domain of NBS1 does not yield a G2/M DNA-damage checkpoint defect. NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild-type, R28A or K160 mutant NBS1, respectively, were left untreated or irradiated at 1 and 10 Gy. Cells were harvested 1 h after irradiation, fixed with methanol and stained with an antibody against phosphorylated H3 (P-H3) and propidium iodide. The percentage of P-H3-positive cells was determined by fluorescence-activated cell sorting analysis. In this graph, three independent experiments (each performed in triplicate) are summarized. The error bars represent the standard deviation. NBS1, Nijmegen breakage syndrome 1.

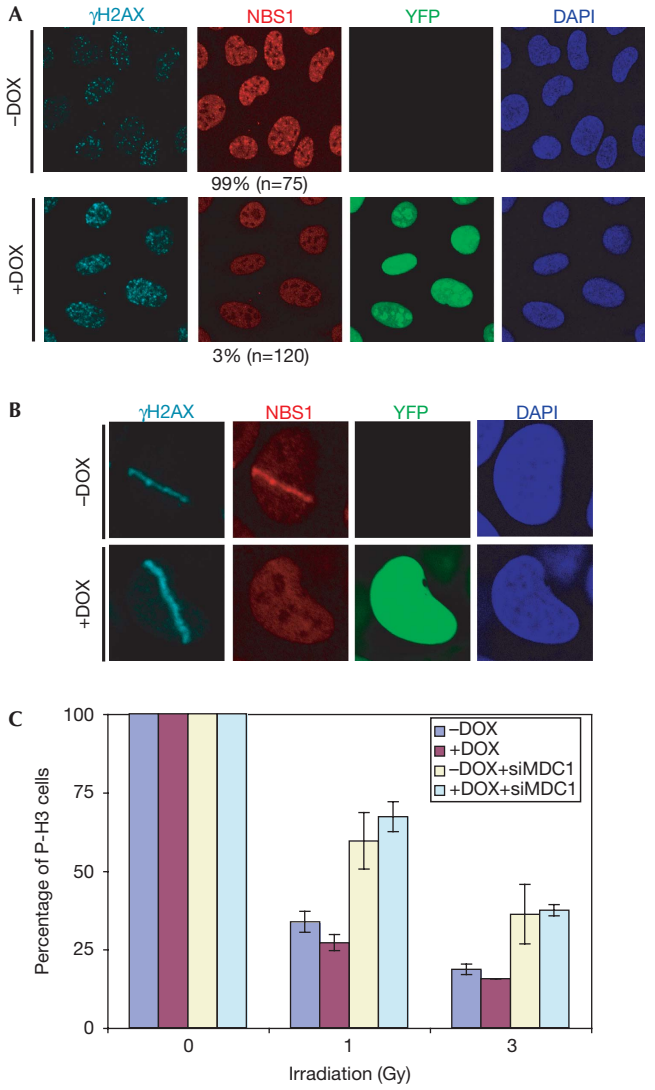

Fig 4 | Experimental uncoupling of the MRN complex from damaged chromatin does not trigger a G2/M checkpoint defect. (**A**) Nuclear foci formation of NBS1 in inducible U2OS YFP-BRCT-overexpressing cells after irradiation at 5 Gy. Non-induced cells (top) and YFP-BRCT-expressing cells (bottom). (**B**) Microlaser-induced DNA-damage recruitment analysis of NBS1 in inducible U2OS YFP-BRCT-overexpressing cells. Non-induced cells (top) and YFP-BRCT-expressing cells (bottom). (**C**) Overexpression of the MDC1 BRCT domains does not trigger a G2/M checkpoint defect. Expression of YFP-BRCT fusion protein was induced 8 h before irradiation (+DOX). Mock-induced cells acted as the control (−DOX). Depletion of endogenous MDC1 by siRNA (siM) partly abrogated the G2/M checkpoint regardless of whether or not MDC1 is proficient for γH2AX binding. The error bars represent the standard deviation. DAPI, 4′,6-diamidino-2-phenylindole; DOX, doxocyclin; MDC1, mediator of the DNA damage checkpoint 1; MRN, MRE11–RAD50–NBS1 complex; NBS1, Nijmegen breakage syndrome 1; siRNA, small interfering RNA; YFP, yellow fluorescent protein.

unknown why such a divalent binding mechanism has evolved, but it is interesting to note that mutation in the NBS1 FHA domain triggers a G2/M checkpoint defect, whereas mutation in the BRCT tandem domain does not. This suggests that besides phosphorylated MDC1, the NBS1 FHA domain might have an additional, as yet unidentified, binding partner that mediates G2/M checkpoint activation in response to DSBs. While this paper was under revision, it was shown that *Schizosaccharomyces pombe* Ctp1, a protein that is involved in the resection of DSBs in the S and G2 phases of the cell cycle, interacts directly with the yeast Nbs1 FHA domain in a mechanism that involves CK2-dependent phosphorylation of SDT-like motifs in Ctp1 (Lloyd *et al*, 2009; Williams *et al*, 2009). Thus, CtIP, the human orthologue of Ctp1, might be a promising candidate for an additional NBS1 interaction partner. CtIP was shown previously to be required for efficient induction of the G2/M DNA-damage checkpoint (Yu & Chen, 2004). Furthermore, human CtIP also contains a region that comprises several conserved CK2 consensus sites; indeed, this region is phosphorylated efficiently by CK2 *in vitro* (F.H. & M.S., unpublished observation). However, whether or not these putative CK2 sites in CtIP interact with human NBS1 to mediate the G2/M DNA-damage checkpoint remains to be established.

METHODS

Cell lines and plasmids. NBS-iLB1 cells stably expressing wild-type and K160M mutant NBS1 were generated by retroviral transduction. The YFP-BRCT-expressing U2OS cell line was described by Stucki *et al* (2005). The human MDC1 GST-SDT construct was described by Spycher *et al* (2008). The MDC1(800) fragment was cloned into a modified pcDNA3.1-Flag mammalian expression vector (Invitrogen, Eugene, OR, USA). Myc-NBS1 was subcloned into a pFastBac transfer vector (Invitrogen) to

generate recombinant NBS1 baculoviruses and into pLPCX (Clontech, Mountain View, CA, USA) to generate retroviral particles, respectively. Point mutations were introduced by using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA).

Single-cell analysis. DSBs in defined nuclear volumes were induced by laser micro-irradiation using an MMI CELLCUT system containing a 355 nm UVA laser (55 Hz; Molecular Machines & Industries, Glattbrugg, Switzerland). Cells were stained with antibodies against human Nbs1 (Novus, Littleton, CO, USA) and γH2AX (Upstate, Temecula, CA, USA). Images were captured by using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 40× (oil immersion, NA 1.25) objective.

Biochemical analysis. GST pulldown assays were performed by mixing 5 μg of GST-fusion proteins with a standard TNT reaction and 5 μg of purified MRN, respectively. For co-immunoprecipitation, human embryonic kidney 293T cells were co-transfected with a Flag-tagged fragment of MDC1 (1–800 amino acids) and Myc-tagged Nbs1 constructs. Anti-Flag(M2)-beads (Sigma-Aldrich, St Louis, MO, USA) were used to immunoprecipitate proteins from total cell extract. All samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting.

G2/M checkpoint analysis. G2/M checkpoint analysis of NBS fibroblasts was performed as described by Spycher *et al* (2008). See the supplementary information online for details.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIALS

METHODS

Cell extraction and protein purification

Hela nuclear extract was purchased from Cilbiotech (Mons, Belgium). MDC1-GST-SDT fragment was affinity purified on Glutathione-Sepharose (GE Healthcare Biosciences). Recombinant MRN purification from Sf9 cells was described (Spycher et al, 2008). For in vitro-translation of full-length NBS1, the TNT system (Promega) was used.

Microirradiation and single-cell analysis

In order to generate DSBs in defined nuclear volumes laser microirradiation was performed with a MMI CELLCUT system containing a 355 nm UVA laser (55 Hz, Molecular Machines & Industries, Switzerland) coupled to an Olympus IX71 microscope station and focused through an LUCPLFLN 40X objective. The MMICELLTOOLS software with MMIUVCUT plug-in assisted the laser operation using an energy output of 50%. Prior to laser irradiation, cells were grown on coverslips in cell culture dishes in the presence of 10 μM BrdU (Bromodeoxyuridine; Sigma) for 24 h. Coverslips were transferred into LabTek chamber slides (Nunc) and mounted on the microscope stage for irradiation. After irradiation, cells were placed back in the incubator for 30-60 min before fixation.

Biochemical analysis

For GST pull down assays, purified GST-fusion proteins (5 μg) were mixed with 1/5 volume of a standard TNT reaction and 5 μg of purified MRN, respectively.

Where indicated, GST fusion proteins were pre-treated with 100 U of CK2 (New England BioLabs). The mixture was incubated at 4°C for 30 min to allow binding. Glutathione sepharose beads were added and the suspension was incubated for further 60 min. The beads were washed with buffer (50mM Tris pH 7.5, 120 mM NaCl, 1 mM DTT, 0.2% NP-40) and resuspended in SDS loading buffer.

For co-immunoprecipitation, HEK 293T cells were co-transfected with a Flag-tagged fragment of MDC1 (1-800 aa) and Myc-tagged Nbs1 constructs. Cells were lysed in lysis buffer (25 mM Tris pH 7.5, 40 mM NaCl, 2 mM $MgCl_2$, 0.5 % NP-40, protease and phosphatase inhibitors, 25 U/ml benzonase (Novagen)) and incubated for 30 min at 4 °C. The concentration of NaCl was increased to 450 mM and incubated for another 30 min at 4 °C. After centrifugation, extracts were diluted to 100 mM NaCl, added to pre-blocked anti-flag(M2)-beads (Sigma) and incubated for 3 h at 4 °C. The beads were washed with IP-buffer (25 mM Tris pH 7.5, 100 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM DTT, 0.5 % NP-40, protease and phosphatase inhibitors) and resuspended in SDS loading buffer. All samples were analyzed by SDS PAGE and immunoblotting.

Checkpoint analysis

Cells were irradiated with a Faxitron X-ray cabinet at the indicated doses during the exponential growth phase. 1h later, cells were harvested, fixed with 70% ethanol/PBS and incubated over night at -20°C. After permeabilization with 0.25% Triton/PBS, cells were stained with anti-phospho-histone H3 (Upstate), followed by secondary anti-FITC (Jackson) or Alexa 700 (Invitrogen) antibodies and propidium iodide. Data were acquired with a Becton Dickinson flow cytometer (NSB

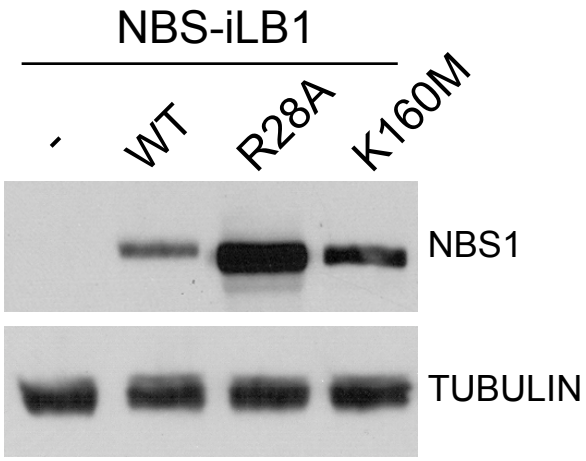
fibroblasts) or a Beckham Coulter CyAn ADP 9 Color flow cytometer (U2OS YFP-BRCT cell line).

Supplementary Figure S1

(A) NBS1 expression profile of NBS-iLB1 fibroblasts stably transduced with wild type NBS1, R28A NBS1 and K160M NBS1.

(B) Two independent clones of NBS-iLB1 fibroblasts stably transduced with wild K160M mutant NBS1 were left untreated or irradiated with 1 Gy and 10 Gy. Cells were harvested 1 hour after irradiation, fixed with methanol and stained with an antibody against phosphorylated H3 (P-H3) and propidium iodine. The percentage of P-H3 positive cells was determined by FACS analysis. NBS-iLB1 parental cells and NBS1-iLB1 cells stably transduced with wild type NBS1 served as negative and positive controls, respectively. Error bars represent standard deviation.

A


NBS-iLB1
-
WT
R28A
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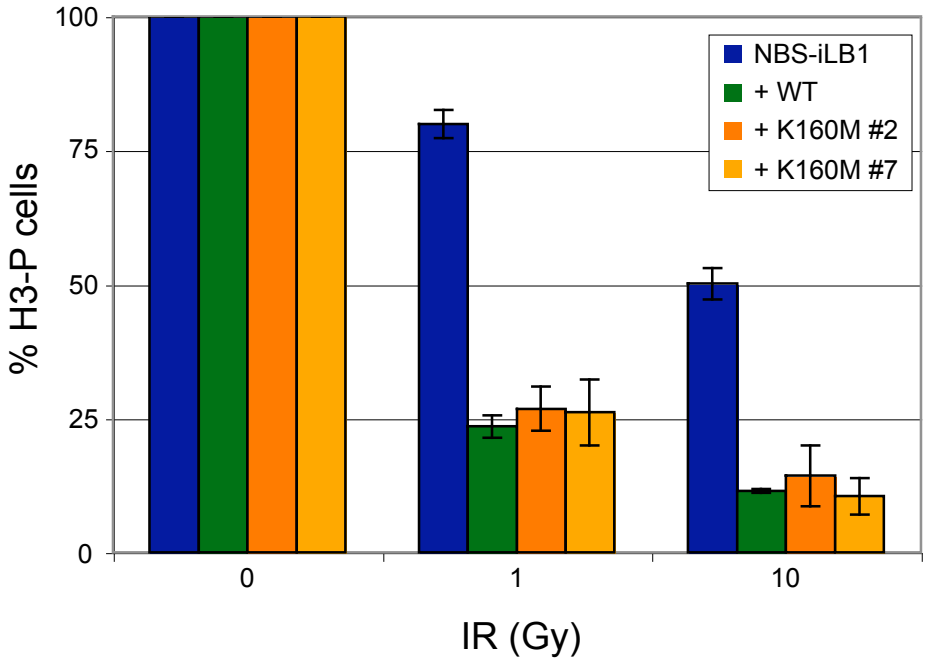
B


100
75
50
25
0
% H3-P cells
0
1
10
IR (Gy)
NBS-iLB1
+ WT
+ K160M #2
+ K160M #7

5.4 Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BRCA1/2	Breast cancer susceptibility gene 1/2
BRCT	BRCA1 C-terminal
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CHK1/2	Checkpoint protein
CK2	Casein kinase 2
CPT	Camptothecin
CtBP	Carboxyl-terminal binding protein
CtIP	CtBP interacting protein
DDR	DNA damage response
DSB	Double-strand break
FHA	Forkhead-associated
GST	Glutathione S-transferase
HNE	HeLa nuclear extract
HR	Homologous recombination
IR	Ionizing radiation
IRIF	Ionizing radiation induced foci
Mb	Megabases
MDC1	Mediator of DNA damage checkpoint 1
MMEJ	Microhomology-mediated end joining
MRN	MRE11-RAD50-NBS1
MS	Mass spectrometry
NBS1	Neijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
NLS	Nuclear localisation signal
NMR	Nuclear magnetic resonance
OHT	Hydroxytamoxifen
PIKK	Phosphatidylinositole(3)-like kinase
RDS	Radioresistent DNA synthesis
ROS	Reactive oxygen species
SDT	Ser-Asp-Thr
SSB	Single-strand break
UV	Ultraviolet light
γH2AX	H2AX phosphorylated on Ser139

5.5 Curriculum vitae

Name	Flurina Judith Hari
Date of Birth	19th April 1982
Place of origin	Adelboden BE
Nationality	Swiss

Education

Since 11/2007	**PhD in Biochemistry, University of Zurich, Switzerland** PD. Dr. Manuel Stucki, Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Switzerland: „Chromatin Changes at Sites of DNA Double-Strand Breaks“
10/2002 - 03/2007	**Diploma in Biology, ETH Zurich, Switzerland** Focus on Biochemistry, Molecular Biology, Cell Biology, Immunology and Genetics Diploma Thesis with Prof. Dr. U. Kutay, Institute of Biochemistry, ETH Zurich: "Human Rio Kinases: Trans-acting Factors Involved in Ribosome Biogenesis"
08/1995 - 01/2002	**Matura Typus B, Kantonsschule Zürcher Oberland Wetzikon, Switzerland** Focus on languages (German, English, French and Latin)

Scientific Publications

Hari, F.J., Spycher, C., Jungmichel, S., Pavic, L., and Stucki, M. (2010). A divalent FHA/BRCT-binding mechanism couples the MRE11-RAD50-NBS1 complex to damaged chromatin. EMBO Rep *11*, 387-392.

Larsen, D.H., Poinsignon, C., Gudjonsson, T., Dinant, C., Payne, M.R., Hari, F.J., Danielsen, J.M., Menard, P., Sand, J.C., Stucki, M.*, et al.* (2010). The chromatin-remodeling factor CHD4 coordinates signaling and repair after DNA damage. J Cell Biol *190*, 731-740.

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